CHAPTER-I, SECTION-4

Design and synthesis of novel triazole and isoxazoline based

*bis*-heterocycles as potential immunopotentiators

(Development of a novel synthetic methodology)
1.4.1. Introduction

Synthetic chemistry has had a great past and its overall impact on science has been remarkable. Particularly the past few decades have seen fascinating developments in synthetic organic chemistry. Chemists are constantly working to discover new, efficient and improved reaction processes for the synthesis of compounds with desired characteristics. Environmental concerns and atom economy have inspired synthetic chemists to search for the development of new, cleaner and efficient chemical transformation methodologies, or modifications in the established conventional synthetic pathways so as to ensure minimal or no use of toxic chemicals for the eco-friendly and cost friendly synthesis of intended substances. In this regard, recent advent of solid and liquid phase synthesis has provided efficient tools for the generation of library of structurally related compounds. The greatest advantage of such methodologies is that a controlled and rapid synthesis of a large number of compounds can be performed in a short time with less practical complications. Rational screening and analysis of the resulting library of compounds increase the chances of discovering pharmacologically important compounds or otherwise better leads for the same. Solution phase chemistry, though limited in scope for generation of a large number of compounds during the same operation, can also be important in this regard as it can provide stereochemically diverse compounds, screening of which can also result in better leads.

Till date, synthetic chemists have developed several highly selective procedures which allow the preparation of complex molecules with excellent regio, chemo, diastereo and enantioselectivities.1 Usual procedure for the synthesis of organic compounds through these procedures involves a stepwise formation of individual bonds in the target molecule. However, it would be much more desirable if one could form several bonds in a single go without isolating the intermediates, changing reaction conditions, or adding reagents.2 It is obvious that in such synthetic procedures, the consumption of solvents, reagents, adsorbents and energy, and production of waste will be lesser in comparison to those for stepwise pathways.

Aromatic heterocycles have been very often used as valuable synthetic templates for the preparation of a variety of new compounds with specific biological or material properties. In recent years, attention has been increasingly paid to the synthesis of
aromatic \textit{bis}-heterocyclic compounds, which exhibit various biological activities including antibacterial, immunostimulatory, fungicidal, tuberculostatic and plant growth regulative properties.\textsuperscript{3} On account of their diverse applications and pharmacological activities particularly in terms of their immunostimulatory effects,\textsuperscript{4,5} \textit{bis}-heterocycles especially those with triazoles/isoaxazolines as their components have received a considerable attention from synthetic chemists. Literature reports related to heterocycles- isoxazolines and triazoles envisage that conjugates of these moieties can prove to be very useful immunopotentiators with potential to demonstrate a synergistic activity of two pharmacophoric groups.

\textbf{1.4.2. Present work}

Inspired by the literature reports related to biological activities of isoxazolines and triazoles, we attempted to develop an efficient, low cost and eco-friendly strategy for the synthesis of biologically active conjugates of these moieties.

From our literature survey exercise we concluded that conventional procedure for the synthesis of isoxazolines involves a multistep process, requiring the generation and subsequent cyclization of nitrile oxides. Some common approaches practiced for these two main steps in the synthesis of isoxazolines reported in literature are:

1. The Mukaiyama method\textsuperscript{6} is based on the reaction of primary nitro-alkanes with phenyl isocyanate in presence of a base like Et\textsubscript{3}N. This method is tedious giving moderate yields of the isoxazolines and besides phenyl isocyanate being hazardous; the replacement of this method is highly desirable.

\begin{equation}
\begin{array}{c}
\text{R-NO}_2 + \text{N=C=O} \\
\xrightarrow{\text{Cat. Et}_3\text{N}} \\
\text{R-\textit{I}-N-O + CO}_2 + \text{N=O} \\
\end{array}
\end{equation}

2. The T. Shimizu method\textsuperscript{7} in which nitroalkane is treated with ethyl carbonochloridate (ClCOOC\textsubscript{2}H\textsubscript{3}) and benzenesulfonyl chloride (C\textsubscript{6}H\textsubscript{5}SO\textsubscript{2}Cl) in presence of Et\textsubscript{3}N, does not give good yield.

\begin{equation}
\begin{array}{c}
\text{R-NO}_2 + \xrightarrow{\text{ClCO}_2\text{Et or PhSO}_2\text{Cl}} \text{Cat. Et}_3\text{N} \\
\xrightarrow{\text{Cat. Et}_3\text{N}} \\
\text{R-\textit{I}-N-O} \\
\end{array}
\end{equation}
3. The B. Singh method\(^8\) in which aromatic oximes are treated with PhICl\(_2\) in presence of pyridine or Et\(_3\)N to yield respective nitrile oxides can be used, only for the synthesis of aromatic oximes that too with low yields.

4. The method of choice and most frequently used is one involving base mediated dehydrohalogenation\(^9\) of hydroxymoyl halides obtained by the reaction of aldoximes with oxidants like NCS, NBS, \(t\)-BuOCl, NaOCl and 1-chlorobenzotriazole, etc.

5. The E. Ryu method\(^10\) in which hydroxymoyl chloride upon treatment with an acrylate in presence of KF or NaF using DCM as a solvent yields isoxazoline. Use of alkali fluorides in this method makes the method less attractive.

6. Cycloaddition reaction of hydroxymoyl chloride with acrylates in presence of Al\(_2\)O\(_3\) or AgOAc\(^11\). Estimated yield for this method is 72% only.

7. S. Kanemasa method\(^12\) wherein hydroxymoyl chloride undergoes cycloaddition reaction with acrylates in presence of BuLi, EtMgBr or Et\(_2\)Zn. However the metal-complexed nitrile oxide is a less reactive species.
8. It has also been envisaged that 1,3-dipolar addition of fulminic acid to enone system can yield isoxazolines. However, the generation of fulminic acid is often troublesome and leads to explosion if metal fulminates are used.

9. 1,3-dipolar cycloaddition of carbethoxyformonitrileoxide (CEFNO) which is generated in situ by treatment of ethyl chloro oximido acetate with aqueous sodium bicarbonate has also been reported. Moerch et al., had earlier investigated the reaction of 16-dehydropregnenolone acetate (16-DPA) with ethyl chloro-oximido acetate and sodium bicarbonate and reported stereospecific formation of product giving isoxazoline derivatives.

In present work, a new method for in situ generation of nitrile oxide and its 1,3-dipolar cycloaddition to yield isoxazoline derivatives has been attempted. We investigated 1,3-dipolar cycloaddition reaction of various in situ generated nitrile oxides (dipoles) over the dipolorophiles known as acrylates. Acrylates of varying chain length were introduced in order to study the effect of chain length on immunostimulating activity. The present method of synthesizing isoxazolines through in situ generation of nitrile oxides from the reaction of aldoximes with NCS without the use of a base or a catalyst or any external reagent giving high yield of the desired substituted isoxazolines under simple reaction conditions is first of its kind to be reported in this direction. In present approach, the reaction takes place regioselectively at the unsaturated double bond of the acrylate moiety resulting in the regioselective synthesis of the desired 3,5-disubstituted isoxazoline derivatives. A series of bis-heterocyclic compounds (Table 1) was synthesized and their immunopotentiating activity explored both under in vitro and in vivo biological conditions. The resulting novel derivatives of isoxazolines conjugated with triazoles proved to be potent immunopotentiators.

Aromatic azide was prepared from the corresponding aniline (1a) (Scheme 1). Diazotization of aniline followed by the nucleophilic displacement of azonium ion with azide anion resulted in the formation of aromatic azide (1b). 1,3-dipolar cycloaddition reaction of azide with propargyl alcohol in toluene yielded alcohols (1c and 1d) which selectively got oxidized to their respective aldehydes (1e and 1f) by activated MnO₂. To the neutralized solution of hydroxylamine hydrochloride (NH₂OH.HCl), aldehyde (1e) was added to afford pure oxime (1g). Oxime, when treated with N-chlorosuccinimide in DMF led to the synthesis of corresponding
chlorooxime which led to *in situ* generation of nitrile oxide without the addition of a base. 1,3-dipolar cycloaddition reaction of *in situ* generated nitrile oxide with acrylate yielded corresponding 3,5-disubstituted isoxazoline carboxylate (1). We observed the formation of a single regio-isomer essentially due to high regioslectivity of reaction involving nitrile oxide cycloaddition to acrylates. The reaction was found to be general with regard to acrylates of varying chain length and substituted triazoles bearing electron donating or electron withdrawing groups on the aromatic ring.

![Scheme 1: Synthesis of bis-heterocycles encompassing triazoles and isoxazolines.](image-url)
1.4.3. Experimental Section

1.4.3.1. Synthesis

The experimental details pertaining to the synthesis of aimed compounds in this study are given as under:

**Typical procedure for the synthesis of Methyl 3-(1-phenyl-1H-1,2,3-triazol-4-yl)-4,5-dihydroisoxazole-5-carboxylate (1):**

*(1-Phenyl-1H-1,2,3-triazol-4-yl)methanol (1c):*

To the aromatic azide (1b) (0.05 g, 0.4 mmol) in toluene (30 mL), formed by the diazotization of aniline (1a), was added propargyl alcohol (0.0067 g, 0.12 mmol). The reaction mixture was allowed to reflux at 110 °C for 18 h. After completion of the reaction (monitored by TLC), the reaction mixture was evaporated to dryness. The reaction was neat and afforded pure *(1-Phenyl-1H-1,2,3-triazol-4-yl)methanol (1c)* (94%) and *(1-Phenyl-1H-1,2,3-triazol-5-yl)methanol (1d)* (<5%). The mixture was used without purification in the next step.

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 4.91 (s, 2H), 7.50-7.78 (m, 5H), 7.98 (s, 1H).

Mass (ESI-MS): 213.9 (M$^+$ + K).


**Structural determination**

Formation of 1,4-disubstituted and 1, 5-disubstituted triazoles was established through the characteristic chemical shift values of the triazolyl protons in 1,4-disubstituted triazoles (5-CH) at $\delta = 7.98-8.62$ in contrast to the appearance of 4-CH signal at $\delta = 7.70-7.80$ in case of 1,5-disubstituted triazoles.$^{16,17}$

**1-Phenyl-1H-1,2,3-triazole-4-carbaldehyde (1e):**

To a mixture of 1c and 1d in CH$_2$Cl$_2$ (0.048 g, 0.274 mmol), was added activated MnO$_2$ (0.238 g, 2.73 mmol). The reaction mixture was allowed to stir for 24 h at ambient temperature. After completion of the reaction (monitored by TLC), the reaction mixture was filtered, evaporated to dryness and finally subjected to column chromatography [silica gel 230-400 mesh as stationary phase, hexane: EtOAc; (8:2) as mobile phase] to afford aldehyde (1e) as a crystalline white solid (86%). 1f was also isolated but the yield was only 1-2%.
1H NMR (200 MHz, CDCl₃): δ 7.55-7.76 (m, 5H), 8.53 (s, 1H), 10.24 (s, 1H).

Mass (ESI-MS): 211.9 (M⁺ + K).

C, H, N analysis for C₉H₇N₃O:

1-Phenyl-1H-1,2,3-triazole-4-carbaldehyde oxime (1g):
Hydroxylamine hydrochloride (0.022 g, 0.32 mmol) was dissolved in water and neutralized with NaOH. To the neutralized solution of hydroxylamine hydrochloride, 1e (0.047 g, 0.271 mmol) was added and reaction mixture stirred for 1 h at ambient temperature. Excess of water was added to the reaction mixture and the organic compound extracted with EtOAc (2 × 30 mL). The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated under vacuum to afford pure oxime (1g) in 99% yield.

1H NMR (200 MHz, CDCl₃): δ 7.46-7.81 (m, 5H), 8.28 (s, 1H), 8.39 (s, 1H), 8.78 (s, 1H).

Mass (ESI-MS): 211 (M⁺ + Na).

C, H, N analysis for C₉H₈N₄O:
Calculated C, 57.44; H, 4.28; N, 29.77. Found C, 57.49; H, 4.21; N, 29.73.

Methyl 3-(1-phenyl-1H-1,2,3-triazol-4-yl)-4,5-dihydroisoxazole-5-carboxylate (1):
1-Phenyl-1H-1,2,3-triazole-4-carbaldehyde oxime (1g) (0.046 g, 0.024 mmol) was dissolved in DMF (10 mL). N-chlorosuccinimide (0.041 g, 0.031 mmol) was added to the above solution at ambient temperature followed by the immediate addition of methyl acrylate (0.023 g, 0.026 mmol) and the reaction mixture stirred for 1 h. Excess of water was added and the organic compound extracted with Et₂O (3 × 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 afford bis- heterocycle (1) in pure form.
Table 1: Synthesis of various bis-heterocycles encompassing triazoles and isoxazolines.

<table>
<thead>
<tr>
<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>
</tr>
</thead>
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<td><img src="image7.png" alt="Compound 7" /></td>
<td>92</td>
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<tr>
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<tr>
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<td><img src="image9.png" alt="Compound 9" /></td>
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<td>91</td>
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<td>92</td>
<td><img src="image11.png" alt="Compound 11" /></td>
<td>90</td>
</tr>
<tr>
<td><img src="image6.png" alt="Compound 6" /></td>
<td>90</td>
<td><img src="image12.png" alt="Compound 12" /></td>
<td>90</td>
</tr>
</tbody>
</table>

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

**Structural determination**

The regio-specific formation of 3,5-disubstituted isoxazolines was established through the characteristic chemical shift values as reported in literature with multiplets at 5.07-5.34 ppm for the C5 protons and 3.80-3.93 ppm for C4 protons.  \(^{18}\)
1.4.3.2. Spectral data

Methyl-3-(1-phenyl-1H-1,2,3-triazol-4-yl)-4,5-dihydroisoxazole-5-carboxylate (1):

![Methyl-3-(1-phenyl-1H-1,2,3-triazol-4-yl)-4,5-dihydroisoxazole-5-carboxylate (1)](image)

White solid; mp: 83-84 °C.

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 3.84 (s, 3H), 3.88-3.90 (m, 2H), 5.21-5.32 (dd, 1H, $J_1 = 14.23$ Hz, $J_2 = 7.72$ Hz), 7.51-7.58 (m, 3H), 7.76 (d, 2H, $J = 7.94$ Hz), 8.46 (s, 1H).

$^{13}$C NMR (500 MHz, CDCl$_3$): $\delta$ 39.39, 52.90, 77.58, 120.70, 120.73, 129.43, 129.95, 136.45, 138.27, 170.40, 171.44.

IR (KBr, cm$^{-1}$): 689, 760, 815, 884, 961, 1002, 1028, 1165, 1238, 1311, 1351, 1437, 1466, 1504, 1560, 1596, 1711, 1725, 1748, 2957, 3137, 3225.

Mass (ESI-MS): 295 (M$^+$ + Na).

C, H, N analysis for C$_{13}$H$_{12}$N$_4$O$_3$: Calculated C, 57.35; H, 4.44; N, 20.58. Found C, 57.32; H, 4.48; N, 20.53.

Ethyl-3-(1-phenyl-1H-1,2,3-triazol-4-yl)-4,5-dihydroisoxazole-5-carboxylate (2):

![Ethyl-3-(1-phenyl-1H-1,2,3-triazol-4-yl)-4,5-dihydroisoxazole-5-carboxylate (2)](image)

White solid; mp: 75-76 °C.

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 1.27 (t, 3H, $J = 4.97$ Hz), 3.88 (q, 2H, $J = 3.03$ Hz), 3.92 (m, 2H), 5.04-5.15 (dd, 1H, $J_1 = 14.18$ Hz, $J_2 = 7.94$ Hz), 7.50-7.55 (m, 3H), 7.77 (d, 2H, $J = 7.75$ Hz), 8.47 (s, 1H).

$^{13}$C NMR (500 MHz, CDCl$_3$): $\delta$ 14.16, 39.50, 62.14, 77.82, 120.83, 129.46, 130.00, 136.52, 138.43, 171.27, 177.81.
IR (KBr, cm⁻¹): 644, 691, 761, 815, 851, 1036, 1182, 1238, 1311, 1367, 1426, 1504, 1531, 1622, 1709, 1771, 2852, 2924, 3440.

Mass (ESI-MS): 309.16 (M⁺ + Na).


Butyl-3-(1-phenyl-1H-1,2,3-triazol-4-yl)-4,5-dihydroisoxazole-5-carboxylate (3):

White solid; mp: 86-87 °C.

¹H NMR (200 MHz, CDCl₃):  δ 0.94 (t, 3H, J = 7.19 Hz), 1.37 (m, 2H), 1.67 (m, 2H), 3.87-3.93 (m, 2H), 4.21 (t, 2H, J = 6.32 Hz), 5.21-5.32 (dd, 1H, J₁ = 14.01 Hz, J₂ = 7.71 Hz), 7.50-7.57 (m, 3H), 7.77 (d, 2H, J = 7.35 Hz), 8.39 (s, 1H).

¹³C NMR (500 MHz, CDCl₃):  δ 13.66, 19.03, 30.49, 39.54, 65.99, 77.80, 120.79, 120.99, 129.50, 120.99, 136.49, 138.31, 178.69, 178.80.

IR (KBr, cm⁻¹): 648, 690, 761, 821, 843, 935, 1003, 1190, 1293, 1372, 1396, 1417, 1466, 1503, 1693, 1771, 1827, 2848, 2955, 3075, 3157.

Mass (ESI-MS): 353.11 (M⁺ + K).

tert-Butyl-3-(1-phenyl-1H-1,2,3-triazol-4-yl)-4,5-dihydroisoxazole-5-carboxylate (4):

White solid; mp: 92-93 °C.

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 1.51 (s, 9H), 3.81-3.84 (m, 2H), 5.07-5.18 (dd, 1H, $J_1 = 15.89$ Hz, $J_2 = 7.89$ Hz), 7.48-7.54 (m, 3H), 7.76 (d, 2H, $J = 7.79$ Hz), 8.46 (s, 1H).

$^{13}$C NMR (500 MHz, CDCl$_3$): $\delta$ 27.84, 29.51, 39.25, 78.21, 82.78, 120.48, 120.64, 129.27, 129.83, 136.41, 138.47, 149.76, 168.82, 177.78.

IR (KBr, cm$^{-1}$): 666, 689, 755, 821, 867, 894, 919, 1038, 1160, 1227, 1248, 1294, 1366, 1463, 1506, 1646, 1709, 1751, 2358, 2851, 2919, 3086, 3134.

Mass (ESI-MS): 353.20 (M$^+ +$ K).

C, H, N analysis for

$C_{16}H_{18}N_4O_3$: Calculated C, 61.14; H, 5.77; N, 17.82. Found C, 61.11; H, 5.76; N, 17.89.

Methyl-3-{1-(4-methoxyphenyl)-1H-1,2,3-triazol-4-yl}-4,5-dihydroisoxazole-5-carboxylate (5):

White solid; mp: 81-82 °C.

$^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 3.79 (s, 3H), 3.87 (m, 2H), 3.92 (s, 3H), 5.17-5.31 (dd, 1H, $J_1 = 17.44$ Hz, $J_2 = 9.03$ Hz), 7.12 (d, 2H, $J = 8.08$ Hz), 7.46 (d, 2H, $J = 7.58$ Hz), 8.61 (s, 1H).

$^{13}$C NMR (500 MHz, CDCl$_3$): $\delta$ 39.39, 52.90, 56.44, 77.58, 113.54, 120.70, 120.73, 129.42, 129.95, 170.40, 171.22, 177.91.

IR (KBr, cm$^{-1}$): 645, 815, 914, 961, 1001, 1164, 1238, 1314, 1364, 1424, 1465, 1507, 1714, 2853, 2924, 2958, 3223.
Mass (ESI-MS): 324.9 (M+ + Na).

C, H, N analysis for C₁₄H₁₄N₄O₄:
Calculated C, 55.63; H, 4.67; N, 18.53. Found C, 55.69; H, 4.62; N, 18.58.

Ethyl-3-[1-(4-methoxyphenyl)-1H-1,2,3-triazol-4-yl]-4,5-dihydroisoxazole-5-carboxylate (6):

White solid; mp: 93-94 °C.

¹H NMR (200 MHz, CDCl₃): δ 1.33 (t, 3H, J = 7.09 Hz), 3.89 (m, 2H), 3.92 (s, 3H), 4.28 (q, 2H, J = 7.10 Hz), 5.15-5.28 (dd, 1H, J₁ = 17.08 Hz, J₂ = 9.05 Hz), 7.12 (d, 2H, J = 8.12 Hz), 7.46 (d, 2H, J = 7.20 Hz), 8.61 (s, 1H).

¹³C NMR (500 MHz, CDCl₃): δ 14.13, 39.53, 56.05, 62.03, 77.67, 112.37, 121.29, 124.72, 130.25, 130.68, 170.03, 171.21, 177.53.

IR (KBr, cm⁻¹): 651, 852, 962, 1016, 1164, 1249, 1295, 1238, 1425, 1542, 1718, 2853, 2923, 3231.

Mass (ESI-MS): 338.9 (M⁺ + Na).

C, H, N analysis for C₁₅H₁₆N₄O₄:
Calculated C, 56.96; H, 5.10; N, 17.71. Found C, 56.94; H, 5.16; N, 17.75.

Butyl-3-[1-(4-methoxyphenyl)-1H-1,2,3-triazol-4-yl]-4,5-dihydroisoxazole-5-carboxylate (7):

White solid; mp: 82-83 °C.

¹H NMR (200 MHz, CDCl₃): δ 0.90 (t, 3H, J = 7.32 Hz), 1.35-1.46 (m, 2H), 1.61-1.68 (m, 2H), 3.83-3.92 (m, 2H), 3.92 (s, 3H), 4.22 (t, 2H, J = 6.39 Hz), 5.15-5.28 (dd, 1H, J₁ = 17.72
Hz, $J_2 = 8.40$ Hz), 7.12 (d, 2H, $J = 7.71$ Hz), 7.46 (d, 2H, $J = 8.30$ Hz), 8.61 (s, 1H).

$^{13}$C NMR (500 MHz, CDCl$_3$): δ 14.13, 19.02, 29.59, 39.60, 56.04, 62.03, 77.66, 112.36, 120.93, 121.28, 130.25, 130.67, 170.03, 171.22, 177.03.

IR (KBr, cm$^{-1}$): 652, 817, 963, 1226, 1250, 1297, 1329, 1426, 1654, 1717, 2853, 2923, 2955, 3397.

Mass (ESI-MS): 367 ($M^+ + Na$).

C, H, N analysis for
C$_{17}$H$_{20}$N$_4$O$_4$: Calculated C, 59.29; H, 5.85; N, 16.27. Found C, 59.26; H, 5.81; N, 16.22.

tert-Butyl-3-{[1-(4-methoxyphenyl)-1H-1,2,3-triazol-4-yl]-4,5-dihydroisoxazole-5-carboxylate (8):}

White solid; mp: 95-96 °C.

$^1$H NMR (200 MHz, CDCl$_3$): δ 1.51 (s, 9H), 3.83-3.89 (m, 2H), 3.92 (s, 3H), 5.13-5.28 (dd, 1H, $J_1 = 18.24$ Hz, $J_2 = 9.05$ Hz), 7.12 (d, 2H, $J = 8.12$ Hz), 7.46 (d, 2H, $J = 7.21$ Hz), 8.61 (s, 1H).

$^{13}$C NMR (500 MHz, CDCl$_3$): δ 27.84, 39.25, 56.04, 78.21, 82.78, 113.55, 120.48, 120.64, 129.27, 129.83, 168.82, 177.78.

IR (KBr, cm$^{-1}$): 645, 758, 814, 962, 1022, 1161, 1312, 1370, 1424, 1461, 1502, 1710, 2853, 2923, 2956, 3235, 3404.

Mass (ESI-MS): 367 ($M^+ + Na$).

C, H, N analysis for
C$_{17}$H$_{20}$N$_4$O$_4$: Calculated C, 59.29; H, 5.85; N, 16.27. Found C, 59.23; H, 5.89; N, 16.25.
Methyl-3-{1-(4-nitrophenyl)-1H-1,2,3-triazol-4-yl}-4,5-dihydroisoxazole-5-carboxylate (9):

![Chemical structure](image)

White solid; mp: 109-110 °C.

$^1$H NMR (200 MHz, CDCl$_3$): δ 3.79 (s, 3H), 3.84-3.91 (m, 2H), 5.20-5.34 (dd, 1H, $J_1 = 18.99$ Hz, $J_2 = 8.79$ Hz), 8.06 (d, 2H, $J = 9.10$ Hz), 8.47 (d, 2H, $J = 9.04$ Hz), 8.67 (s, 1H).

$^{13}$C NMR (500 MHz, CDCl$_3$): δ 37.39, 52.41, 78.00, 120.21, 120.76, 121.54, 138.96, 140.10, 145.74, 161.01, 170.16.

IR (KBr, cm$^{-1}$): 643, 855, 1032, 1186, 1294, 1345, 1458, 1520, 1705, 2853, 2924, 2956, 3407.

Mass (ESI-MS): 317 (M$^+$).

C, H, N analysis for C$_{13}$H$_{11}$N$_5$O$_5$: Calculated C, 49.22; H, 3.49; N, 22.07. Found C, 49.25; H, 3.48; N, 22.02.

Ethyl-3-{1-(4-nitrophenyl)-1H-1,2,3-triazol-4-yl}-4,5-dihydroisoxazole-5-carboxylate (10):

![Chemical structure](image)

White solid; mp: 108-109 °C.

$^1$H NMR (200 MHz, CDCl$_3$): δ 1.34 (t, 3H, $J = 7.18$ Hz), 3.85-3.90 (m, 2H), 4.24-4.35 (q, 2H, $J = 7.07$ Hz), 5.18-5.31 (dd, 1H, $J_1 = 18.39$ Hz, $J_2 = 8.56$ Hz), 8.02 (d, 2H, $J = 9.04$ Hz), 8.47 (d, 2H, $J = 9.02$ Hz), 8.57 (s, 1H).

$^{13}$C NMR (500 MHz, CDCl$_3$): δ 14.12, 39.10, 62.19, 78.39, 120.32, 120.90, 125.46, 139.43, 147.00, 149.59, 161.01, 170.76.

IR (KBr, cm$^{-1}$): 644, 752, 860, 1030, 1092, 1160, 1309, 1345, 1402, 1522, 1651, 1715, 2924, 3133.
Chapter - I, Section - 4

Mass (ESI-MS): 353.9 (M⁺ + Na).
C, H, N analysis for C₁₄H₁₃N₅O₅:

Butyl-3-{1-(4-nitrophenyl)-1H-1,2,3-triazol-4-yl}-4,5-dihydroisoxazole-5-carboxylate (11):

White solid; mp: 108-109 °C.

¹H NMR (200 MHz, CDCl₃):
δ 0.91 (t, 3H, J = 7.25 Hz), 1.42 (m, 2H), 1.68 (m, 2H), 3.85-3.90 (m, 2H), 4.23 (t, 2H, J = 7.25 Hz), 5.18-5.31 (dd, 1H, J₁ = 18.99 Hz, J₂ = 9.24 Hz), 8.03 (d, 2H, J = 8.90 Hz), 8.47 (d, 2H, J = 8.91 Hz) 8.59 (s, 1H).

¹³C NMR (500 MHz, CDCl₃): δ 13.66, 19.02, 30.50, 39.18, 65.99, 77.99, 120.29, 120.90, 125.71, 139.37, 140.58, 149.56, 169.74, 170.12.

IR (KBr, cm⁻¹): 643, 857, 1034, 1091, 1199, 1344, 1388, 1458, 1524, 1650, 1742, 2854, 2924, 3133, 3382.

Mass (ESI-MS): 382.0 (M⁺ + Na).
C, H, N analysis for C₁₆H₁₇N₅O₅:
Calculated C, 53.48; H, 4.77; N, 19.49. Found C, 53.46; H, 4.73; N, 19.46.

tert-Butyl-3-{1-(4-nitrophenyl)-1H-1,2,3-triazol-4-yl}-4,5-dihydroisoxazole-5-carboxylate (12):

White solid; mp: 102-103 °C.
Chapter - I, Section - 4

1H NMR (200 MHz, CDCl3): \( \delta 1.52 \) (s, 9H), 3.80-3.84 (m, 2H), 5.07-5.21 (dd, 1H, \( J_1 = 18.94 \) Hz, \( J_2 = 9.04 \) Hz), 8.02 (d, 2H, \( J = 8.97 \) Hz), 8.97 (d, 2H, \( J = 8.88 \) Hz), 8.59 (s, 1H).

13C NMR (500 MHz, CDCl3): \( \delta 26.53, 43.53, 77.75, 119.10, 119.75, 122.92, 136.47, 140.51, 141.27, 164.12, 170.75. \)

IR (KBr, cm\(^{-1}\)): 683, 858, 888, 1032, 1156, 1288, 1343, 1402, 1523, 1597, 1742, 2852, 2921, 3133.

Mass (ESI-MS): 382 (M\(^+\)+Na).

C, H, N analysis for C\(_{16}\)H\(_{17}\)N\(_5\)O\(_5\):
Calculated C, 53.48; H, 4.77; N, 19.49. Found C, 53.46; H, 4.73; N, 19.46.

1.4.3.3. Biological experiments

All the \textit{in vitro} and \textit{in vivo} experiments were performed in a similar manner as discussed in \textit{section 3} of this chapter.

1.4.4. Results

Initially all the compounds were tested for their possible role in lymphocyte proliferation under \textit{in vitro} conditions. On account of the higher activity observed for compounds 7 and 8 in \textit{in vitro} studies, these were selected for further \textit{in vivo} evaluation. Many assays were used to observe their influence on antibody production (IgM and IgG), DTH reaction, T-Cell subtypes (CD4 and CD8), splenocyte proliferation \textit{ex vivo} (T cell and B cell proliferation), cytokine production (IL-2, IFN-\( \gamma \), IL-4), NO (macrophage) production and toxic effects. Levamisole, a known immunostimulator reported to augment the antibody response,\(^{19}\) was given orally as positive control, at a dose of 2.5 mg/kg body weight.

**Effect of test compounds on \textit{in vitro} lymphocyte proliferation by MTT assay**

All the compounds show dose-related increase or decrease of titre (Figure 1). Among all the screened moieties, compounds 1-8 increased lymphocyte proliferation to a good extent in comparison to levamisole. Maximum stimulation was shown by compound 8 at a dose of 10 \( \mu \)g/mL. Compounds 6 and 7 were also effective at a dose of 10 \( \mu \)g/mL. Compounds 9-12 produced a dose related decrease in lymphocyte proliferation. While compounds 11 and 12 showed some stimulation at a dose of 0.1 \( \mu \)g/mL, the compounds 9 and 10 were found to be least active.
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. Both the compounds showed dose-related increase of titre (Figure 2). However, compound 8 was found to be more effective in increasing the antibody titre to a higher extent 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 and a highest stimulatory effect was observed at a dose of 0.1 mg/kg. Compound 7 enhanced antibody production more than levamisole at doses 0.01 mg/kg and 0.1 mg/kg body weight. From the data it can be seen that the induced increase in IgG titre is more than that in IgM titre.

![Effect on HA titre](image)

**Figure 2:** Effect of compounds 7 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 7 and 8 on SRBC induced DTH reaction was assessed in mice at various doses and the results are summarized (Figure 3). Compound 8 was found to be a potential candidate in enhancing DTH response at all doses with a maximum achieved at 0.1 mg/kg. Even at lower dose of 0.001 mg/kg, DTH response was found to be
higher in comparison to levamisole. Both these two compounds induced better DTH response at 24 h study followed by 48 h and 72 h respectively.

![Effect on DTH response](image)

**Figure 3:** Effect of compounds 7 and 8 on DTH response. Data are expressed as mean ± S.E. of five observations of right 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 splenocyte proliferation ex vivo (T and B cell proliferation)**

The effect of compounds 7 and 8 on splenocyte proliferation under *ex vivo* conditions was assessed in mice following various doses and the results are summarized (**Figure 4**). Out of the two compounds evaluated, compound 8 induced significantly higher T and B cell proliferation at all doses and maximum at a dose of 0.1 mg/kg. At all the doses (0.001 mg/kg, 0.01 mg/kg and 0.1 mg/kg) proliferation values are higher than that observed with levamisole. Compound 7 also showed better activity than the standard.
Figure 4: Splenocyte proliferation expressed as the absorption at 570 nm. Data are mean ± SE of six animals. *P < 0.05; **P < 0.01; ***P < 0.001 compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test).

Effect on spleen T-cell subtyping

Spleen single cell suspension (10^6 cell/mL) was studied for CD4^+/CD8^+ T-cell subtypes by anti-CD4 and CD8 monoclonal antibodies conjugated with fluoreseine-isothio-cyanate (FITC) and phycoerthyrin (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 (Table 2).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>CD4^+ T-Cell (%)</th>
<th>CD8^+ T-Cell (%)</th>
<th>CD4/CD8 Ratio</th>
<th>Spleen CD4^+ Content (x10^7)</th>
<th>Spleen CD8^+ Content (x10^7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (vehicle)</td>
<td>20.7 ± 0.90</td>
<td>13.3 ± 0.34</td>
<td>1.56 ± 0.09</td>
<td>2.70 ± 0.12</td>
<td>1.47 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Levamisole</td>
<td>2.5</td>
<td>30.8 ± 1.30a</td>
<td>18.3 ± 0.53a</td>
<td>1.68 ± 0.06a</td>
<td>1.48 ± 0.10a</td>
<td>1.38 ± 0.10a</td>
</tr>
<tr>
<td>Compound 7</td>
<td>0.001</td>
<td>27.8 ± 0.59</td>
<td>18.8 ± 0.38</td>
<td>1.47 ± 0.07</td>
<td>1.36 ± 0.06</td>
<td>1.27 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>33.2 ± 0.61</td>
<td>19.6±0.38b</td>
<td>1.69±0.07</td>
<td>1.53 ± 0.06</td>
<td>1.42± 0.05</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>40.1 ± 0.70</td>
<td>21.3 ± 0.46</td>
<td>1.88±0.07</td>
<td>1.82 ± 0.07</td>
<td>1.49 ± 0.05</td>
</tr>
<tr>
<td>Compound 8</td>
<td>0.001</td>
<td>33.3 ± 0.53</td>
<td>19.4 ± 0.34</td>
<td>1.71±0.06</td>
<td>1.51 ± 0.05b</td>
<td>1.43 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>39.6 ± 0.59</td>
<td>20.1 ± 0.36b</td>
<td>1.97±0.06</td>
<td>1.65 ± 0.05</td>
<td>1.49 ± 0.04b</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>44.3 ± 0.66a</td>
<td>21.0 ± 0.46</td>
<td>2.10±0.05a</td>
<td>1.77± 0.06a</td>
<td>1.54± 0.03</td>
</tr>
</tbody>
</table>

Table 2: Effect of different doses of compounds 7 and 8 on spleen T cell subtypes.  
Number of observation = 6  (a) P < 0.01; (b) P < 0.05
Maximum effect of 8 was obtained at 0.1 mg/kg dose, 44.3% CD4+ and 21.0% CD8+ T cells. The respective control values were 20.70% of CD4+ and 13.3% of CD8+ T cells. This shows a significant increase in CD4+ T cell count. Levamisole, a standard T-cell stimulator 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.

**Effect on cytokine release (IL-2, IFN-γ and IL-4)**

In order to understand the specific effects of compounds 7 and 8 on cytokine profiles, characteristic IL-2, IFN-γ and IL-4 were analyzed. Both these compounds stimulated IL-2, IFN-γ and IL-4 release in a dose dependent manner (Figure 5).

![Figure 5: Effect of compounds 7 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).](image)

Compound 8 effectively increased release of cytokines at all doses and with a maximum at dose of 0.1 mg/kg. At a dose of 0.001 mg/kg compounds 7 showed effect
comparable to that of levamisole but higher activity at a dose of 0.1 mg/kg. All together, release of IL-4 was least among the three types of cytokines.

**Effect on NO production**

The compounds 7 and 8 were tested for their possible role in macrophage production. Both the compounds showed dose-dependent increase of macrophage production (Figure 6). Both these compounds stimulated NO production to a greater extent than levamisole. However, compound 8 showed maximum stimulation (at a dose of 0.1 mg/kg) followed by compound 7.

![Graph showing Effect on NO production](image)

**Figure 6:** Effect of compounds 7 and 8 on NO production. Results are expressed in μM. Data are mean ± SE of six animals. *P < 0.05; **P < 0.01; ***P < 0.001 compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test).

**Toxicity studies**

The results from our toxicity studies indicate that the test compounds did not exhibit any toxic effect even at a dose of as high as 100 μg/mL and after 72 h incubation.

**1.4.5. Discussion**

In investigations related to immunostimulating effect, the bis-heterocyclic isoxazoline derivatives synthesized in current study were observed to express a dose dependent impact on different immune cells. Under *in vitro* conditions for lymphocyte proliferation, we observed a dose dependent increase (electron donating/unsubstituted aryl rings of triazole) or decrease (electron withdrawing groups on aryl ring of triazole) of titre. The results from preliminary assays showed that isoxazolines with a suitably substituted non polar hydrocarbon chain along with suitably substituted triazole possess good immunopotentiating activity. Presence of electron donating
groups (OCH₃) on the aryl ring of triazole moiety was found to be highly stimulating followed by unsubstituted aryl ring. Similarly tert-butyl chain was found to be a better substituent on the isoxazoline ring followed by n-butyl, ethyl and methyl chains respectively for the generation of stronger immune response. Since compounds 7 and 8 increased lymphocyte proliferation to a better extent than levamisole, these were chosen for further biological studies under in vivo conditions. Compound 8 was found to be strongly immunopotentiating at all the doses in comparison to compound 7 and levamisole in all in vivo experiments. Compound 7 was more effective than levamisole at doses 0.01 mg/kg and 0.1 mg/kg. From these observations, it can be inferred that a small branched tertiary butyl group on the isoxazoline ring together with an electron donating group (p-OCH₃) on the aryl ring of triazole facilitates interaction of the bis-heterocycle with the target cells. Both the compounds 7 & 8 were effective in increasing splenocyte proliferation, DTH response, cytokine release and NO production in BALB/c mice. 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⁺ values for these compounds clearly indicate the immunogenic response through MHC-class II pathway. The induction of cytokines after treatment with the test compounds in these experiments could be attributed to the release of significant amounts of NO. Here again the observed CD4⁺ and CD8⁺ values were in conformity with the antibody response, which qualifies compounds 7 and 8 as the highest active molecules and hence can be advocated as powerful alternatives to currently available immunostimulators in both prophylactic and/or therapeutic applications.

1.4.6. Conclusion

In conclusion a novel, facile and expedient synthetic pathway for the synthesis of novel unsymmetrical biologically important bis-heterocycles encompassing triazole and isoxazoline moieties is presented in this section. The synthesized compounds were found to express a significant immunostimulating activity both in in vivo and in vitro conditions. The results presented besides providing clues for establishing target specificity in these compounds are expected to be very useful for future studies aimed at arriving a better understanding of the mode of action of such compounds in immunostimulation.
1.4.7. References


5. a) Knysh YH, Parchenko VV, Panasenko OI, Kaplaushenko AH, Makovyk YV, Kulish SM, Hotsulia AS, Izdepskiy VY, Kyrychko BP, Mysyk OH. 1,2,4-Triazole-3-ylthioacetic acid derivatives exhibiting the antioxidant, hepatoprotective and immunopotentiating activity. Patent No 87184, 25.06.2009.


17. Wang ZX, Qin HL. Regioselective synthesis of 1,2,3-triazole derivatives *via* 1,3-dipolar cycloaddition reactions in water. *Chem Commun* 2003:2450-2451.


$^1$H NMR spectrum of compound 8
ESI-MS spectrum of compound 8

[M⁺ + Na]
$^{13}$C NMR spectrum of compound 8
DEPT NMR spectrum of compound 8
IR spectrum of compound 8
$^{1}$H NMR spectrum of compound 10
ESI-MS spectrum of compound 10
$^{13}$C NMR spectrum of compound 10
DEPT NMR spectrum of compound 10