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Mechanochemical synthesis, simultaneous double cycloaddition reactions of bisnitrones and potential anticancer activities of the bis cycloadducts

Bhaskar Chakraborty*[a,b], Esmita Chettri[a] & Sushma Tamang Pradhan[a]

[a] Organic Chemistry Laboratory, Sikkim Government College (NBBDC), Gangtok 737 102, Sikkim, India

[b] Academic visitor, School of Chemistry, Cardiff University, Park Place, Cardiff CF10 3AT, Wales, UK

E-mail: bhaskargtk@yahoo.com

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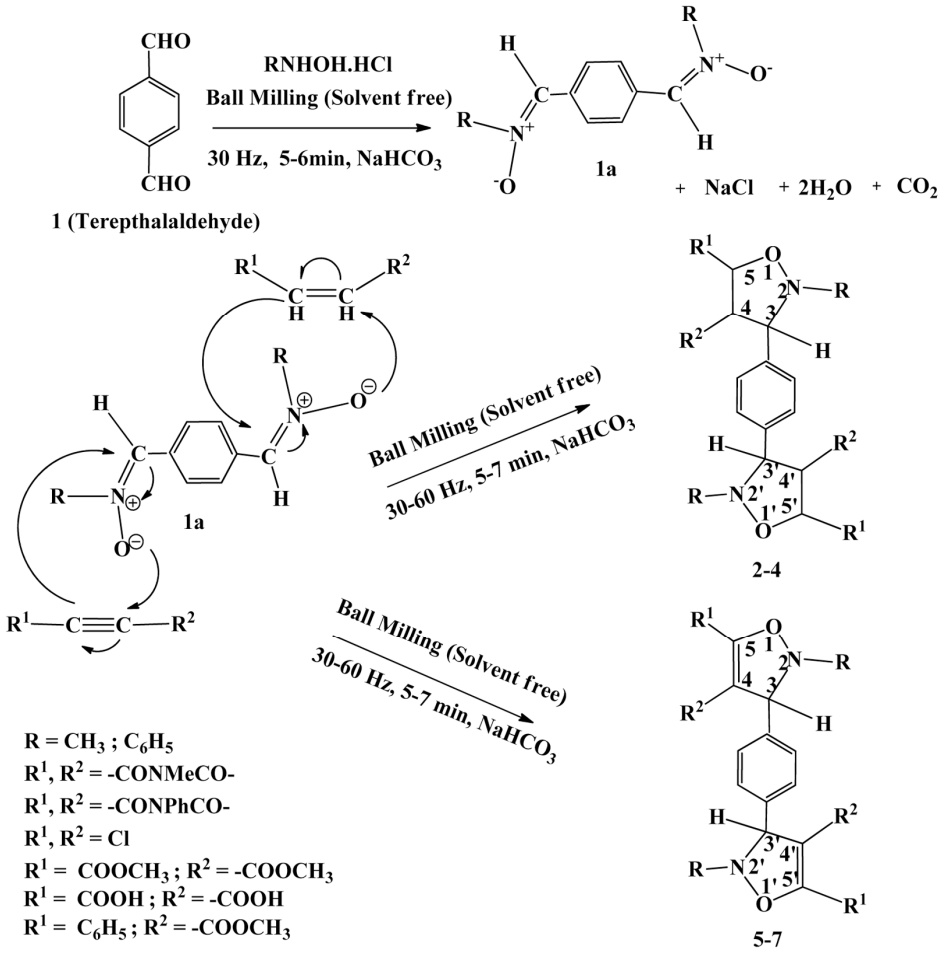
Solid phase synthesis of bisnitrones and cycloaddition reactions of some bisnitrones using mechanochemical procedure has been reported. Change in reaction rate and yields of the bisnitrones as well as bis cycloadducts are the key factors which is highly encouraging after comparing microwave and coventional cycloaddition procedures. This study reports synthesis of terephthalaldehyde and glyoxal derived bis-nitrones and their cycloaddition reactions with activated alkenes and electron deficient alkynes along with significant anticancer activities of a few bis-cycloadducts.

Keywords: Mechanochemistry, Bisnitrones, Intermolecular cycloaddition reaction, Bis-isoxazolidine, Isoxazolines, Anticancer activity

Perhaps one of the most important reaction intermediates in synthetic organic chemistry is nitrone as far as the construction of the synthetically significant 5-membered oxygen-nitrogen heterocycles are concerned[1]. The synthesis, isolation cycloaddition reactions of nitrones with activated olefins and alkynes are always a point great interest not only because of the synthetic potentials of cycloadducts (isoxazolidine and isoxazoline derivatives) but also for their excellent biological activities[2-4]. Though various reports of this chemistry is widely known but synthesis and cycloaddition reactions of bisnitrones and bis cycloadducts has not reported too many[5-7]. It could be because of the difficulties associated in the synthetic procedures, isolation and further applications in this chemistry. Keeping in mind the widespread applications of different nitrones in cycloaddition reaction, we would like to report in this communication synthesis of some important stable bis (isoxazolidine) and bis (isoxazoline) derivatives using mechanochemical procedures (Scheme 1, Table 1)[8,9] along with the significant anticancer activities of few bis cycloadducts. We have also compared with the synthetic procedures of bis-cycloadducts with microwave and conventional methodologies and found significant acceleration in reaction rate and yield of the bis-cycloadducts in mechanochemical procedures. Mechanochemical procedures involving ball-milling technique are very popular nowadays for its many environmental friendly aspects and hence attracted the attention of mainly synthetic organic chemists[8]. Conducting organic synthesis under environment friendly conditions is always a challenge therefore ball-milling technique is frequently used as a lucrative methodology in the synthesis of important medicinally compounds. So, this methodology is recommended as an alternative to conventional procedures *viz*, heating, microwave irradiation and sonication[9].

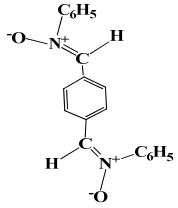
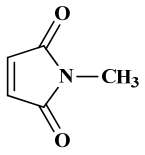
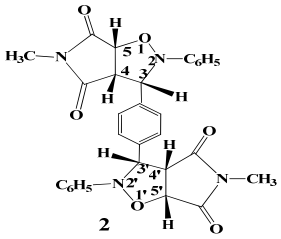
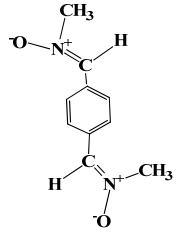
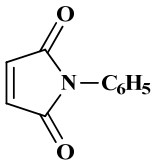
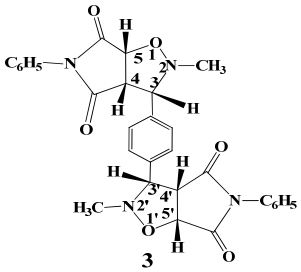
Terephthalaldehyde and glyoxal derived bis nitrones are less-known group of nitrones but they are of special interest in organic synthesis for many reasons. Like majority of usual nitrones, bisnitrones (terepthalaldehyde bisnitrones) are stable (melting points ranges between 74°C to 142°C), therefore utilization of these nitrones in cycloadditions reaction becomes very easy. Moreover, bis-cycloadducts found to exhibit significant anticancer activities. Hence, these bis-nitrones may be regarded as an important precursor for the synthesis of new anticancer drugs which could attract organic and medicinal chemists in research.

Our group has reported many environment friendly (green chemistry) procedures already in nitrone cycloaddition reactions and further applications of cycloadducts[10-17] and now we have decided to advance



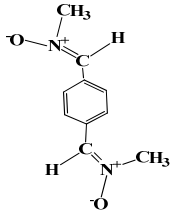
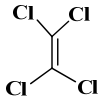
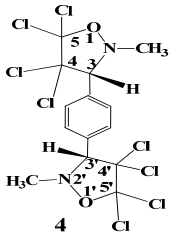
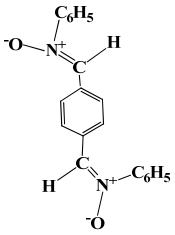
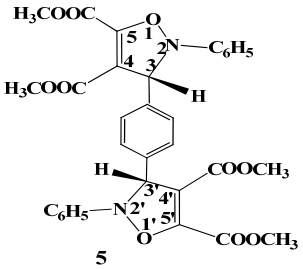
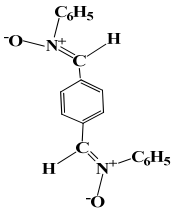
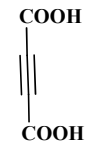
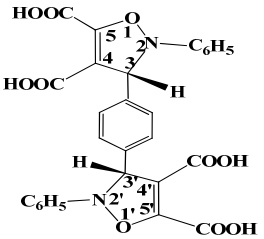
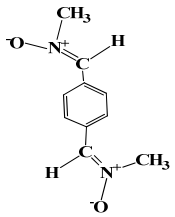
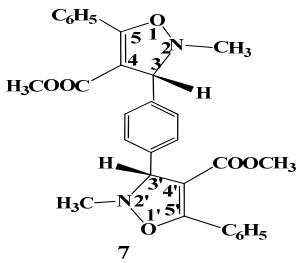
Scheme 1 — Synthesis of bis-(isoxazolidine and isoxazoline derivatives from terepthaldehyde derived bisnitrone

Table 1 — 1,3-Dipolar cycloaddition reaction of terephthalaldehyde derived bisnitrones with alkenes and alkynes

Entry	Bisnitrone[a] (**1a**)	Alkene and alkynes	Bis(isoxazolidine and isoxazolines)[b] (**2-7**)	Time (min)	Yield[c] (%)
1			 **2**	5 (5)	95 (92)
2			 **3**	5 (6)	95 (90)

(*Contd*.)

Table 1 — 1,3-Dipolar cycloaddition reaction of terephthalaldehyde derived bisnitrones with alkenes and alkynes (*Contd*.)

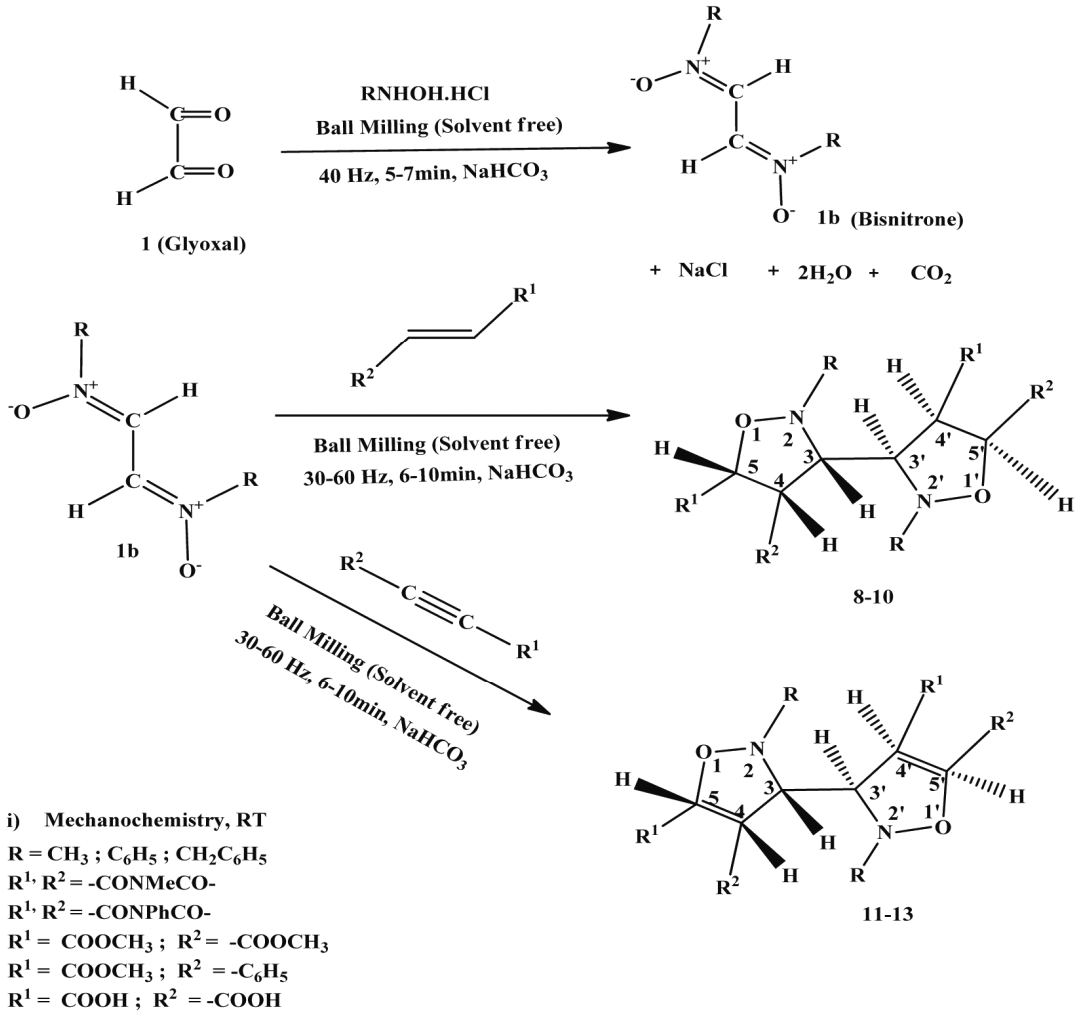
Entry	Bisnitrone[a] (**1a**)	Alkene and alkynes	Bis(isoxazolidine and isoxazolines)[b] (**2-7**)	Time (min)	Yield[c] (%)
3			 **4**	6 (not done)	93
4			 **5**	5 (8)	92 (88)
5			 **6**	8 (not done)	92
6			 **7**	8 (not done)	90

[a] Reaction conditions: bisnitrone (1 mmol), alkenes and alkynes (2 equivalent), ball-milling (40-70Hz), at 30-40°C
[b] All products were characterized by IR, ^{1}H and ^{13}C NMR and MS spectral data.
[c] Isolated yield after purification. Figures in parentheses indicate reactions performed in MWI (at 90-130°C)

the procedures in the synthesis and simultaneous double cycloaddition reactions of bisnitrones using terepthalaldehyde and glyoxal as precursors following mechanochemical procedures (Scheme 1 and Scheme 2, Table 1 and Table 2)[10,11].

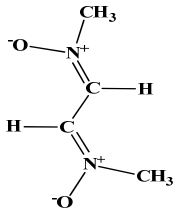
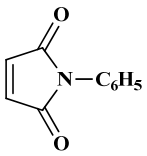
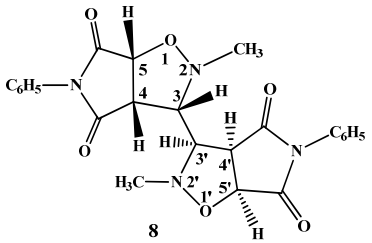
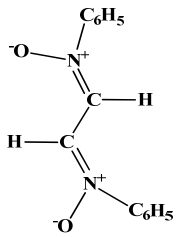
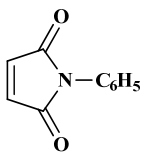
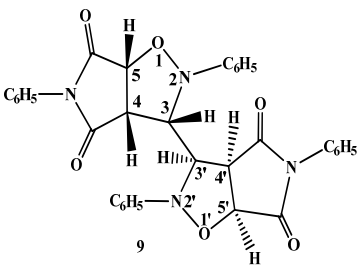
Experimental Section

Mechanochemical procedure (ball-milling) was performed in Retsch MM500 digital mixer mill, Retsch GmbH, 42781, Hann, Germany. Bruker DRX 300 spectrometer (300 MHz, FT NMR) was used for recording 1H NMR spectra and TMS was used as internal standard. Same instrument was used for recording 13C NMR spectra at 75 MHz. The coupling constants (J) are expressed in Hz. Perkin-Elmer RX 1-881 machine was used for recording IR spectra as film or as KBr pellets for all the molecules. Jeol SX-102 (FAB) instrument was used for recording MS spectra. Perkin-Elmer 2400 series. Progress of all the reactions were monitored by TLC using 0.25 mm silica gel plates (Merck 60F254 UV indicator). Column chromatography was performed with silica gel (E.Merck Germany) with 60–200 mesh. The starting materials and reagents used in the reactions were obtained commercially from Sig-Aldrich, E.Merck (Germany) and were used without further purification unless otherwise indicated.



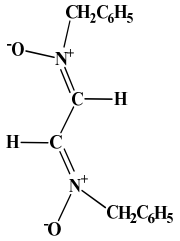
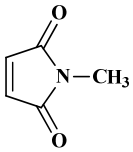
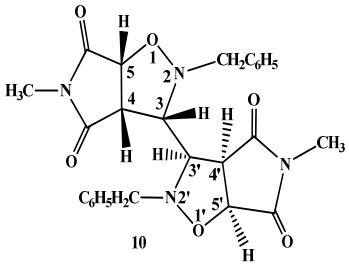
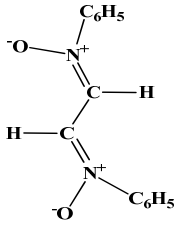
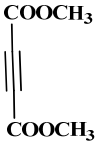
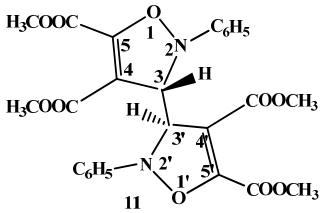
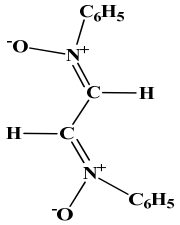
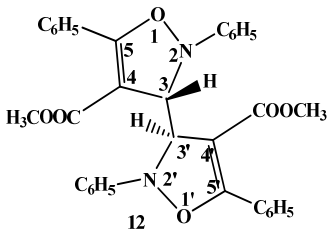
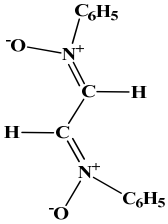
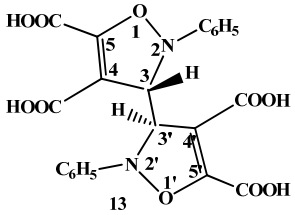
Scheme 2 — Synthesis of bis-(isoxazolidine and isoxazoline derivatives) glyoxal derived bisnitrone

Table 2 — 1,3-Dipolar cycloaddition reaction of glyoxal derived bisnitrones with alkenes and alkynes

Entry	Bisnitrone[a] (**1b**)	Alkene and alkynes	Bis(isoxazolidine and isoxazolines)[b] (**8-13**)	Time (min)	Yield[c] (%)
1			 **8**	6 (6)	94 (90)
2			 **9**	6 (8)	94 (90)

(*Contd.*)

Table 2 — 1,3-Dipolar cycloaddition reaction of glyoxal derived bisnitrones with alkenes and alkynes (*Contd*.)

Entry	Bisnitrone[a] (**1b**)	Alkene and alkynes	Bis(isoxazolidine and isoxazolines)[b] (**8-13**)	Time (min)	Yield[c] (%)
3			 **10**	7 (8)	94 (88)
4			 **11**	8 (10)	92 (86)
5			 **12**	8 (not done)	92 (not done)
6			 **13**	10 (not done)	92 (not done)

[a] Reaction conditions: bisnitrone (1 mmol), alkenes and alkynes (2 equivalent), ball-milling (40-70Hz), at 30-40°C
[b] All products were characterized by IR, ^{1}H NMR, ^{13}C NMR and MS spectral data.
[c] Isolated yield after purification. Figures in parentheses indicate reactions performed in MWI (at 90-130°C)

General procedure I–Mechanochemical synthesis of terephthalaldehyde derived bis nitrones

To a 25 mL stainless steel milling vessel (Retsch MM500 mixer mill digital GmbH, 42781 Haan, Germany) was added 2 stainless steel balls, terephthalaldehyde (670mg, 5 mmol), *N*-phenylhydroxylamine hydrochloride (1.90g, 10mmol, 2 equivalent) and $NaHCO_3$ (1 mmol). A stainless steel milling ball was added and the mixture milled at 40 Hz for 5 min. The formation of bisnitrone was monitored by TLC (R_f = 0.40).After the completion of reaction, CH_2Cl_2 was added. The mixture was then filtered on cotton to remove NaCl. The filtrate was evaporated under vacuum to afford the desired product terepthalaldehyde derived bisnitrone (**1**) as white crystals (90%; m.p: 112°C) without need for further purification. Same methodology was followed for the synthesis of other bisnitrones (R=C_6H_5). Both the bisnitrones were found to be stable and were reacted with various activated alkenes and alkynes in 1,3-dipolar cycloaddition reaction.

Spectroscopic data for nitrone **1a** (R = C_6H_5): UV λ_{max} 248 nm; IR (KBr): 3115 (m), 1650 (m), 1610 (s) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.20 (s, 4H, aromatic carbons), 7.70-6.90 (m, 2×5H), 1.30 (s, 2×1H, -CH=N^+); ^{13}C NMR (75 MHz, $CDCl_3$): δ 145.50 (2 x CH=N^+), 137.44, 137.30, 137.14, 136.97, 135.77, 135. 62 (aromatic carbons), 129.67, 129.52,

128.06 (phenyl carbons linking 2-C-atoms); FAB-MS: *m/z* 316 (M^+), 239, 196, 77.

Spectroscopic data for nitrone **1a** (R = CH_3): UV λ_{max} 232 nm; IR (KBr): 3096 (m). 1660 (m), 1615 (s) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 6.90 (s, 4H, aromatic protons), 4.10 (s, 2×1H, -CH=N^+), 2.34 (s, 2×6H, N-CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 142.30 (2 x CH=N^+), 129.44, 129.25 (phenyl carbons), 24.46 (2×CH_3 carbons); FAB-MS: *m/z* 192 (M^+), 134 (BP), 133.

General procedure II–Mechanochemical synthesis of bis(isoxazolidine andisoxazoline) derivatives from terephthalaldehyde derived nitrones (Table 1; entry 1)

To a 25 mL stainless steel milling vessel containing terepthalaldehyde derived nitrone **1a** (R=C_6H_5; 1 equivalent) was added *N*-methylmaleimide (2 equivalent) and the mixture was milled at 40 Hz for 5 min. After the completion of reaction, as indicated by TLC (R_f= 0.66), CH_2Cl_2 was added and the mixture was filtered on cotton for the removal of NaCl. The filtrate was evaporated under vacuum to afford pure bisisoxazolidine **2** (Table **1**, entry **1**, 95%) as white crystals (m.p.142°C). Same methodology was followed for other substrates depicted in Table 1.

Spectral data of bisisoxazolidine derivatives, 2-4)

(*3R*, 3a*R*, 6a*S*)-Dihydro-3-((3'*S*, 3'a*S*, 6a*R*)--2, 5-diphenyl-4,6-dioxo-2*H*-pyrrolo[3,4-*d*]isoxazol-3-yl)-2', 5'-dimethyl-2*H*-pyrrolo[3,4-*d*]isoxazole-4,6 (5*H*, 6a*H*) dione, 2

2 (entry 1, Table 1): White crystals. Yield 95%. R_f= 0.66; FT-IR (KBr): 3006 (m), 1760 (s), 1670 (s), 1470 (m), 1230 (m), 784 (s) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.88-7.50 (m, 2×5H), 7.20 (s, 4H, aromatic protons), 6.42 (d, *J* = 3.00 Hz, C_5H protons), 6.42 (d, *J* = 3.20 Hz, C_3H protons), 3.80 (s, 6H, 2×N-CH_3), 2.3 (dd, *J* = 3.00 Hz, 2×1H, C_4H protons); ^{13}C NMR ($CDCl_3$): δ 176.70, 175.10 (carbonyl carbons), 135.77, 133.60, 133.42, 133.15, 133.00 (2×C_6H_5, aromatic carbons), 127.55, 127.14 (2×4 aromatic carbons), 73.54 (C_5, $C_{5'}$), 68.60 (C_3, $C_{3'}$), 55.34 (C_4, $C_{4'}$), 24.70, 24.52 (methyl carbons); FAB-MS: *m/z* 534 (M^+), 305, 229 (BP), 154, 90, 77.

(*3R*, 3a*R*, 6a*S*)-Dihydro-3-((3'*S*, 3'a*S*, 6a*R*)--2,5-diphenyl-4,6-dioxo-2*H*-pyrrolo[3,4-*d*]isoxazol-3-yl)-2', 5'-dimethyl-2*H*-pyrrolo[3,4-*d*]isoxazole-4,6(5*H*, 6a*H*) dione, 3

3. (entry 2, Table 1): White crystals. Yield 95%. R_f= 0.64; FT-IR (KBr): 3030 (m), 1765 (s), 1680 (s), 1474 (m), 1235 (m), 780 (s) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.88-7.50 (m, 2×5H), 7.20 (s, 4H, aromatic protons), 6.42 (d, *J* = 3.00 Hz, C_5H protons), 6.42 (d, *J* = 3.20 Hz, C_3H protons), 3.80 (s, 6H, 2×N-CH_3), 2.3 (dd, *J* = 3.00 Hz, 2×1H, C_4H protons); ^{13}C NMR ($CDCl_3$): δ 173.50, 173.25 (carbonyl carbons), 134.60, 134.15, 133.40, 133.00, 132.80 (2×C_6H_5, aromatic carbons), 128.35, 128.40 (2×4 aromatic carbons), 75.20 (C_5, $C_{5'}$), 67.30 (C_3, $C_{3'}$), 57.30 (C_4, $C_{4'}$), 26.55 (methyl carbons); FAB-MS: *m/z* 534 (M^+), 305, 229 (BP), 154, 90, 77.

(*3R*, 3a*R*, 6a*S*)-Dihydro-3-((3'*S*, 3'a*S*, 6a*R*)-2, 5-dimethyl-3,4-dichloro-6-dioxo-2*H*-pyrrolo[3,4-*d*]isoxazol-3-yl)-2',5'-dimethyl-3',4'-dichloro-2*H*-pyrrolo[3,4-*d*]isoxazole-4,6(5*H*, 6a*H*) dione, 4

4. (entry **3**, Table **1**):White solid. Yield 92%. R_f= 0.72; FT-IR (KBr): 3026 (m), 1768 (s), 1677 (s), 1455 (m), 1250 (m), 778 (s) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.58-7.30 (2×dd,4H, aromatic protons), 3.80 (2×3H, N-CH_3), 1.80 (s, 1H, C_3H), 1.40 (s,1H, $C_{3'}H$); ^{13}C NMR ($CDCl_3$): δ 125.76, 125.40 (2×4 aromatic carbons), 72.43 (C_5, $C_{5'}$), 67.30 (C_3, $C_{3'}$), 54.68 (C_4, $C_{4'}$), 27.26 (CH_3 carbons); FAB-MS: *m/z* 528 (M^+), 300, 228, 154.

(*3R*, 3a*R*, 6a*S*)-Dihydro-3-((3'*S*,3'a*S*,6a*R*)-(S)-2-phenyl-2,3-dihydroisoxazole-4,5-methyldicarboxylate-2*H*-pyrrolo[3,4-*d*]isoxazol-3-yl)-2-2'-diphenyl-4'5'-dimethyl-dicarboxylate-2*H*-pyrrolo[3,4-*d*]isoxazole), 5

5. (entry **4**, Table **1**): White gummy mass. Yield 90%. R_f= 0.68; FT-IR (KBr): 3036 (m), 1770 (s), 1685 (s), 1440 (m), 1235 (m), 788 (s) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.95-7.50 (m, 2×5H), 7.20 (dd, 2×2H, 4H, aromatic protons), 3.35 (br, s, 12H, $COOCH_3$ protons), 2.80 (s, H, C_3H proton), 1.30 (s,1H,$C_{3'}H$); ^{13}C NMR ($CDCl_3$): δ 168.70, 168.54 (carbonyl carbons), 135.45, 133.20, 133.13, 133.00, 132.66 (2×C_6H_5, aromatic carbons), 128.48, 128.34 (4 aromatic carbons), 74.40 (C_5, $C_{5'}$), 65.48 (C_3, $C_{3'}$), 53.90 (C_4, $C_{4'}$), 25.40 (CH_3 carbons of $COOCH_3$); FAB-MS: *m/z* 600 (M^+), 446, 338, 262 (BP),, 154, 77.

(*3R*, 3a*R*, 6a*S*)-Dihydro-3-((3'*S*,3'a*S*,6a*R*)-(S)-2-phenyl-2,3-dihydroisoxazole-4,5-dicarboxylic acid-2*H*-pyrrolo[3,4-*d*]isoxazol-3-yl)-2'-diphenyl-4'5'-dicarboxylic acid-2*H*-pyrrolo[3,4-*d*]isoxazole), 6

(entry **5**, Table **1**):White viscous liquid. Yield 90%. R_f= 0.72; FT-IR (KBr): 3020 (m), 1766 (s), 1675 (s), 1450 (m), 1260 (m), 776 (s) cm^{-1}; ^1H NMR ($CDCl_3$): δ 10.10 (s, 4×1H, COOH protons), 7.90 (dd, 2×2H, 4H0, 7.70-7.45 (m,2×5H), 3.28 (s, 1H, C_3H),

2.70 (s, 1H, $C_{3'}H$); ^{13}C NMR ($CDCl_3$): δ 167.55, 167.43 (carbonyl carbons), 137.55, 137.40, 137.32, 137.00, 136.76 (2×C_6H_5, aromatic carbons), 126.30, 126.18 (4 aromatic carbons), 73.65 (C_5, $C_{5'}$), 64.80 (C_3, $C_{3'}$), 52.90 (C_4, $C_{4'}$), 22.17 (C_3 and $C_{3'}$carbons); FAB-MS: *m/z* 544 (M^+), 390, 310 (BP), 154.

(*3R*, 3a*R*, 6a*S*)-Dihydro-3-((3'*S*,3'a*S*,6a*R*)-(S)-2-methyl-2,3-dihydroisoxazole-4,5-phenyl,methyldicarboxylate-2*H*-pyrrolo[3,4-*d*]isoxazol-3-yl)-2'-methyl-4'5'- phenyl, methyl dicarboxylate 2*H*-pyrrolo[3,4-*d*]isoxazol-3-yl), 7

7. (entry **6**, Table **1**):White gummy liquid. Yield 90%. R_f= 0.70; FT-IR (KBr): 3010 (m), 1760 (s), 1680 (s), 1440 (m), 1255 (m), 788 (s) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.90 (dd, 2×2H, 4H), 7.70-7.45 (m, 2×5H), 3.35 (s, 6H, 2×$COOCH_3$), 3.20 (s, 2×1H, C_3H and $C_{3'}H$), 2.70 (s, 6H, N-CH_3 protons); ^{13}C NMR ($CDCl_3$): δ 169.55, 168.43 (carbonyl carbons), 137.55, 137.40, 137.32, 137.00, 136.76 (2×C_6H_5, aromatic carbons), 126.30, 126.18 (4 aromatic carbons), 73.65 (C_5, $C_{5'}$), 64.80 (C_3, $C_{3'}$), 52.90 (C_4, $C_{4'}$), 37.80 (s, 2×3H, $COOCH_3$), 30.15 (s, 6H, N-CH3 protons),22.17 (C_3 and $C_{3'}$carbons); FAB-MS: *m/z* 512 (M^+), 358, 294 (BP), 218, 154.

General procedure II–Mechanochemical synthesis of glyoxal derived nitrones

To a 25 mL stainless steel milling vessel (Retsch MM500 mixer mill digital GmbH,42781 Haan, Germany) was added 2 stainless steel balls, glyoxal (220mg, 3.246mmole), *N*-substitutedhydroxylamines hydrochloride (500mg, 2 equivalent) and $NaHCO_3$ (1 mmol). A stainless steel milling ball was added and the mixture milled at 30 Hz for 6 min. The formation of bisnitrone was monitored by TLC (R_f = 0.36). After the completion of reaction, CH_2Cl_2 was added. The mixture was then filtered on cotton to remove NaCl. The filtrate was evaporated under vacuum to afford the desired product bisnitrone as white gummy mass (86%). The bisnitrone was found to be unstable and decomposes at RT if kept for more than 48 hours. Therefore, cycloadditions were performed without further delay once the bisnitrone is isolated. Same methodology was followed for the synthesis of other bisnitrones (R = CH_3; C_6H_5; $CH_2C_6H_5$).

Spectroscopic data for nitrone **1b** (R = CH_3): UV λ_{max} 233 nm; IR (KBr): 1635 (m), 1610 (s) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 3.36 (d, 1H, *J* = 3.00 Hz, -CH=N^+), 3.00 (s, 6H, 2×CH_3, N^+-CH_3), 2.84(d, *J* = 2.20 Hz, -CH=N^+), ^{13}C NMR (75 MHz, $CDCl_3$): δ 141.60 (CH=N^+), 140.94 (CH=N^+), 24.74, 24.70 (N^+-CH_3).

Spectroscopic data for nitrone **1b** (R = C_6H_5): UV λ_{max} 242 nm; IR (KBr): 3110 (m). 1630 (m), 1610 (s) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.90-7.25 (m, 2×5H), 6.58 (d, 1H, *J* = 3.10 Hz, -CH=N^+), 6.48(d, *J* = 2.20 Hz, -CH=N^+); ^{13}C NMR (75 MHz, $CDCl_3$): δ 148.27 (CH=N^+), 131.48 (CH=N^+), 130.44, 130.24, 129.58, 129.30, 128.36 (2×aromatic carbons).

Spectroscopic data for nitrone **1b** (R = $CH_2C_6H_5$): UV λ_{max} 246 nm; IR (KBr): 3113 (m). 1615 (m), 1625 (s) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.65-7.50 (m, 2×5H), 6.14 (d, 1H, *J* = 4.30 Hz, -CH=N^+), 6.12(d, *J* = 4.70 Hz, -CH=N^+), 2.54 (s, 2×2H, CH_2 protons); ^{13}C NMR (75 MHz, $CDCl_3$): δ 140.60 (CH=N^+), 139.30 (CH=N^+), 131.75, 130.42, 128.50, 128.22, 127.16 (2×aromatic carbons).

General procedure II–Mechanochemical synthesis of bis(isoxazolidine and isoxazoline) derivatives from glyoxal derived nitrones (Table 2, entry 1)

To a 25 mL stainless steel milling vessel containing glyoxal derived nitrone **1a** (R=CH_3; 1 equivalent) was added *N*-methylmaleimide (2 equivalent) and the mixture was milled at 40 Hz for 40 min. After the completion of reaction, as indicated by TLC (R_f= 0.68), CH_2Cl_2 was added and the mixture was filtered on cotton for the removal of NaCl. The filtrate was evaporated under vacuum to afford pure bisisoxazolidines **8** (Table **2**, entry **1**, 94%) as yellowish white crystals. Same methodology was followed for other substrates depicted in Table **2**.

(*3R*, 3a*R*, 6a*S*)-Dihydro-3-(3'*S*, 3'a*S*, 6a*R*)-hexahydro-2-methyl-5-phenyl-4,6-dioxo-2*H*-pyrrolo[3,4-*d*]isoxazol-3-yl)-2'-methyl-5'-phenyl-2*H*-pyrrolo[3,4-*d*]isoxazole-4,6(5*H*, 6a*H*) dione, 8

(entry 1, Table 2): White crystals. Yield 94%. R_f= 0.68; FT-IR (KBr): 2820 (m), 1760 (s), 1675 (s), 1465 (m), 1230 (m), 1125 (s) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.40-7.18 (m, 2×5H), 3.26 (s, 6H, 2×N-CH_3), 2.20 (dd, *J* = 2.10, 2.10 Hz, C_4H and $C_{4'}H$), 1.90 (d, *J* = 2 Hz, 2×C_5H and $C_{5'}H$), 1.66 (dd, *J* = 2.00, 2.00 Hz, C_3H and $C_{3'}H$)); ^{13}C NMR ($CDCl_3$): δ 174.78, 173.12 (carbonyl carbons), 75.80 (C_5, $C_{5'}$), 69.94 (C_3, $C_{3'}$), 56.77 (C_4, $C_{4'}$), 26.63, 26.58 (methyl carbons); FAB-MS: *m/z* 462 (M^+), 231(BP), 154, 77.

(*3R*, 3a*R*, 6a*S*)-Dihydro-3-((3'*S*, 3'a*S*, 6a*R*)-hexahydro-4,6-dioxo-2,5-diphenyl-2*H*-pyrrolo[3,4-*d*]isoxazol-3-yl)-2',5'-diphenyl-2*H*-pyrrolo[3,4-*d*]isoxazole-4,6(5*H*, 6a*H*)dione, 9

(entry **2**, Table 2): White crystals. Yield 94%. R_f= 0.70; FT-IR (KBr): 3025 (m), 2830 (m), 1764 (s),

1660 (s), 1485 (m), 1345 (m), 784 (s) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.60-7.46 (m, 2×5H), 7.40-7.32 (m,2×5H), 3.90 (dd, *J* = 2.20, 2.16 Hz, 2H, 2×C_4H and $C_{4'}$H), 2.20 (dd, *J* = 2.10, 2.10 Hz, 2×1H, C_3H and $C_{3'}$H), 1.90 (d, *J* = 3.00 Hz, C_5H), 1.82 (d, *J* = 3.00 Hz, $C_{5'}$H); ^{13}C NMR ($CDCl_3$): δ 172.40, 172.26 (carbonyl carbons), 138.83, 138.12, 137.94, 137.71, 129.74, 129.70, 129.33, 129.04 (aromatic carbons), 76.15 (C_5, $C_{5'}$), 66.47 (C_3, $C_{3'}$), 55.80 (C_4, $C_{4'}$); FAB-MS: *m/z* 586 (M^+), 293, 292, 216, 154, 77.

(*3R*, 3a*R*, 6a*S*)-2-Benzyl-3-((3'*S*, 3'a*S*, 6a*R*)-2'-benzyl-hexahydro-5-methyl-4,6-dioxo-2*H*-pyrrolo[3,4-*d*]isoxazol-3-yl)-dihydro-5-methyl-2*H*-pyrrolo[3,4-*d*]isoxazole-4,6(5*H*, 6a*H*)dione, 10

(entry **3;** Table **2):** White crystals. Yield 92%. R_f= 0.62; FT-IR (KBr): 3010 (m), 2900 (m), 1760 (s), 1660 (s), 1482 (m), 1340 (m), 780 (s) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.46-7.26 (m, 10H, 2×$CH_2C_6H_5$), 4.37 (d, 2H, *J* = 7.16 Hz, 2×C_5H and$C_{5'}$H)), 3.24 (d, 2H, *J* = 7.14 Hz, 2×C_3H and $C_{3'}$H), 2.89 (dd, br, 2H, 2×C_4H and $C_{4'}$H), 2.60 (s, 6H, 2×N-CH_3 protons), 2.15 (s, 4H, 2×C$H_2C_6H_5$); ^{13}C NMR ($CDCl_3$): δ 177.18, 176.04 (carbonyl carbons), 136.22, 133.12, 130.90, 127.70 (aromatic carbons), 78.67 (C_5, $C_{5'}$), 67.80 (C_3, $C_{3'}$), 56.77 (C_4, $C_{4'}$), 32.05 (benzyl carbons), 30.20 (N-Me carbons); FAB-MS: *m/z* 490 (M^+), 308, 245, 244, 182, 91, 77.

(*3R*, 3a*R*, 6a*S*)-2,5-Diphenyl-2*H*-pyrrolo[3,4-*d*] isoxazol-3-yl)-2', 5'-diphenyl-2*H*-pyrrolo[3,4-*d*] isoxazole-4,6-dimethyldicarboxylate(5*H*, 6a*H*)dione, 11

Colourless gummy liquid. Yield 92%. R_f= 0.70; FT-IR (KBr): 3030 (m), 2815 (m), 1760 (s), 1665 (s), 1480 (m), 1340 (m), 788 (s) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.50-7.30 (m, 10H, 2×(O=C)NC_6H_5), 3.30 (br, s, 12H, 4×$COOCH_3$). 1.67 (d, 2H, *J* = 6.10 Hz, 2×C_3H and $C_{3'}$H); ^{13}C NMR($CDCl_3$): δ 172.40, 172.26, 171.78, 170.60 (carbonyl carbons), 138.83, 138.12, 137.94, 137.71, 129.74, 129.70, 129.33, 129.04 (aromatic carbons), 76.15 (C_5, $C_{5'}$), 66.47 (C_3, $C_{3'}$), 55.80 (C_4, $C_{4'}$), 23.22, 22.58, 22.12, 21.43 (ester methyl carbons); FAB-MS: *m/z* 572 (M^+), 418, 262 (BP), 154, 77.

(3R, 3aR, 6aS)-2,5-Diphenyl-2*H*-pyrrolo[3,4-d]isoxazol-3-yl)-2', 5'-diphenyl-2*H*-pyrrolo[3,4-d]isoxazole-4,6-phenylmethylcarboxylate(5*H*, 6a*H*)dione, 12

12. (entry **5**, Table 2): Pale yellow liquid. Yield 92%. R_f= 0.74; FT-IR (KBr): 3010 (m), 2810 (m), 1755 (s), 166\0 (s), 1485 (m), 1342 (m), 780 (s) cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.80-7.55 (m, 10H, 2×(O=C)NC_6H_5), 7.50-7.15 (m, 10H), 6.70 (d, 1H, *J* = 6.06 Hz, C_3H), 6.60 (d, 1H, *J* = 5.00 Hz, $C_{3'}$H), 3.30 (br, s, 6H, 2×$COOCH_3$); ^{13}C NMR($CDCl_3$): δ 172.80, 172.58 (carbonyl carbons), 137.60, 137.33, 137.14, 136.90, 136.47, 129.45, 129.30, 128.48 (aromatic carbons), 75.50(C_5, $C_{5'}$), 65.40(C_3, $C_{3'}$), 57.74(C_4, $C_{4'}$), 22.90 (ester methyl carbons), 20.12 (N-methyl carbons); FAB-MS: *m/z* 560 (M^+), 280, 154, 77.

(3R, 3aR, 6aS)-2,5-Diphenyl-2*H*-pyrrolo[3,4-d] isoxazol-3-yl)-2', 5'-diphenyl-2*H*-pyrrolo[3,4-d] isoxazole-4,6-dicarboxylic acid(5*H*, 6a*H*)dione, 13

(Table **2;** entry **6):** Pale yellow liquid. Yield 92%. R_f = 0.74; FT-IR (KBr): 3010 (m), 2810 (m), 1755 (s), 166\0 (s), 1485 (m), 1342 (m), 780 (s) cm^{-1}; ^1H NMR ($CDCl_3$): δ 10.80 (br, s, 4H, COOH), 7.74-7.25 (m, 2×5H, phenyl protons), 6.54 (d, 1H, *J* = 3.06 Hz, C_3H), 6.30 (d, 2H, *J* = 2.00 Hz, $C_{3'}$H); ^{13}C NMR ($CDCl_3$): δ 172.80, 172.58(carbonyl carbons), 137.60, 137.33, 137.14, 136.90, 136.47, 129.45, 129.30, 128.48 (aromatic carbons), 75.50(C_5, $C_{5'}$), 65.40(C_3, $C_{3'}$), 57.74(C_4, $C_{4'}$); FAB-MS: *m/z* 468 (M^+), 314, 234, 154, 90, 77.

Results and Discussion

In our new endeavour of bisnitrone synthesis, we have conducted the reactions taking one equivalent of terepthalaldehyde /glyoxal and two equivalents of *N*-substituteddroxylaminehydrochloride along with one equivalent of sodium bicarbonate for the synthesis of bisnitrones **1a** and **1b** (Scheme 1). The synthesized bisnitrones have been used for the double cycloaddition reactions without further purification. Glyoxal derived bisnitrones were found to be moderately stable and therefore we conducted double cycloaddition reactions immediately after its generation while terepthalaldehyde derived bis nitrones were very stable (m.p: 74°C to 142°C) and cycloaddition reactions were performed with dipolarophiles in 1:2 ratio. Usually, heat (30-40°C) and slight pressure is developed during ball-milling procedure in the reaction vessel In our study, best results have been obtained when1:2 ratio of starting materials were used but incomplete conversion to molecules under study were obtained when tried with 1:1 ratios of starting materials. We added sodium bicarbonate in the reaction mixture in the synthesis of bisnitrones because we had observed that it could

activate the *N*-substitutedhydroxylamines. The probable reason could be due to the addition of sodium bicarbonate the reaction mixture becomes faintly alkaline and the liberated HCl is neutralized. Another important feature in mechanochemistry we have observed is that in absence of solid material (sodium bicarbonate), the leads to develop paste or a gum and things do not mix well. Solid material also helps in free flow proceedings in this synthesis. So, we have used, solid sodium bicarbonate for more efficiency in the process and obtained best results. In absence of solid sodium bicarbonate, we found incomplete reaction (as observed in TLC) with the development of gummy or semisolid as products which were recovered using methanol. We have also studied ^{1}H NMR spectrum of the crude products and found no such indications of developments of our target molecules. We have also noticed that at a lower frequency (10-20Hz), the reactions rates were very slow and needed almost 1hr for the completion of the reaction and in few reactions the bisaldehyde and hydroxylamines were also present with the crude products. It could be due to lesser amount of energy per impact is involved and ball-milling procedure with an interval develops lower conversion rates from starting materials to products.

In mechanochemistry, the formation of nitrones were fast and therefore it becomes easy for conducting *in situ* cycloaddition reactions if researchers so desire. But in our study, we wanted to study the stability and characterize the bisnitrones, therefore we had conducted intermolecular cycloaddition reactions of the bisnitrones with various activated double bonded dipolarophiles (maleimides and tetrachloroethylene) as well as electron deficient dipolarophiles (alkynes). After successful study of various reaction conditions and trials, finally we decided to run the cycloaddition reactions in ball-milling process at a frequency of 40-60Hz and found excellent development of bis cycloadducts in 30-40 minutes of ball-milling. Our research group also studied these reactions using acetonitrile (1 mL) as solvent (polar solvent) in ball-milling procedure and found slight less developments and lower yields of bis cycloadducts. The present cycloaddition procedure and the yields of bis cycloadducts were also compared (Table **1** and **2**) with microwave technology (MWI) under solvent-free conditions[18–20]. We found slight lower yields of bisnitrones and bis cycloadducts in MWI. In microwave methodology, for the synthesis of bisnitrones and bis cycloadducts required high temperature (120-130°C) though average time required for the synthesis of bisnitrones and bis cycloadducts were found to be almost same (6-10 min) respectively. In addition, complete conversion of the starting materials (terepthalaldehyde and glyoxal) to our target molecules remains far from the actual target (around 90-95%) yield as found in ball-milling. Probably, it could be due to the degradation of *N*-substitutedhydroxylaminehydrochlorides as MWI procedure has to be conducted at high temperature (120-130°C). In our continuing study, we also studied DMF as solvent in MWI methodology for cycloaddition reactions but yields (bis isoxazolidine and isoxazolines) were found to be 80-85% which were regarded as poor in comparison with ball-milling process.

The newly synthesized molecules (bis isoxazolidine and isoxazoline derivatives) and bis nitrones have been confirmed using ^{1}H NMR spectroscopy[10,11,21,22]. ^{1}H NMR spectrum of the bisisoxazolidine and isoxazoline derivatives (**2-7** and **8-13**) reveals that the structures are expected to be symmetrical in nature. The 3-H, 4-H protons are *cis* orientated in both terepthaldehyde and glyoxal derived cycloadducts as far as their close proximity of coupling constant (*J*) values. In terephthalaldehyde derived cycloadducts, due to the presence of phenyl ring cordinating between two isoxazolidine rings, the interactions between 3-H and 3'-H was not observed whilst coupling between 3-H and 3'-H in glyoxal derived cycloadducts have been observed and the coupling constants have been found to be $J_{3,3'} \sim 3.00$ Hz[12]. Mechanochemical procedure has been found to be completely environment friendly, much faster and produces high yield of products[9]. For example, the reaction between bisnitrone **1a** and *N*-methyl maleimide produces bisisoxazolidine **2** at RT after 26 h in 62% yield in CH_2Cl_2 while 94% yield of the same molecule **2** is obtained in mechanochemical procedure (Table **1**; entry **1**). All the intermolecular cycloadditions of bisnitrone **1a** and **1b** with different alkenes and alkynes follow the general mechanistic pattern of 1,3-dipolar cycloaddition reactions as we found in literature[4,23-25]. Majority of bis cycloadducts have been found to be very stable, can be kept at RT. Prominent molecular ion peak (M^+) and base peaks (BP) has been observed in the MS spectrum in addition to other fragmentations peaks. The 3-H and 4-H proton signals of terephthalaldehyde derived

Table 3 — IC_{50} values (μM) of various bis (isoxazolidine and isoxazoline) derivatives

Compd (μg mL^{-1})	HeLa (Cervical)	MDA-MB-231 (Breast)	MCF-7 (Breast)	A549 (Lung cancer)
2	98	85	97	95
3	55	93	82	56
4	70	94	60	80
5	38	80	32	18
8	73	67	48	20
9	60	08	42	17
Doxorubicin (Standard)	0.8	2.00	0.4	0.6

isoxazolidine rings (**2-4**) appeared as doublet and double doublets respectively (except in case of tetrachloroethylene bis cycloadduct) while in case of bis cycloadducts (**8-10**) 3-H and 3'H, 4-H and 4'H proton signals of both the isoxazolidine rings appeared as double doublets. The C_3H, C_4H and C_5H protons of both the bis isoxazolidine rings (*cis, cis*) of the bis isoxazoline derivatives have been merged expectedly in majority of the cases and appeared as a single signal while few exceptions are also found. Double doublet signals of C_4H protons of bis cycloadducts, the coupling constant values could not be calculated due to their close proximity. Enhanced reaction rates, excellent yields, environment friendly procedures are the important features observed in ball-milling procedure applied for double cycloaddition reactions. The structures of bis cycloadducts (bis isoxazolidine and isoxazoline) were characterized by ^{1}H and ^{13}C NMR, IR and MS spectroscopic data.

Initial study reports of the anticancer study few synthesized bis isoxazolidine and isoxazoline derivatives are also very encouraging. All bisisoxazolidine derivatives (**2-7** and **8-13**)) have been screened for anticancer studies against various breast and lung cells and few of them provided good initial study reports which gives an opportunity to develop new anticancer agents. Screening study (SEM and TEM) on the few novel bisisoxazolidines (tetrachloro bisisoxazolidine and ethylene dicarboxylic acid bis isoxazoline) are going on at present.

Anticancer study

Majority of the newly synthesized heterocycles in recent years are screened for cancer studies[26] as their probability to act as anticancer drugs is very high. Cytotoxicity of the compounds was determined on the basis of measurement of *in vitro* growth inhibition of tumor cell lines[27-29]: A549 derived from human alveolar adenocarcinoma epithelial cells (ATCC No.CCL-180), HeLa derived from human cervical cancer cells (ATCC No. CCL-14), MDA-MB-167 derived from human breast adenocarcinoma cells (ATCC No. HTB-35) and MCF7 derived from human breast adenocarcinoma cells (ATCC No. HTB-40) using the MTT assay. The IC_{50} values (50% inhibitory concentration in μM) are expressed as the average of two independent experiments The effect of bis cycloadducts (**2-13**) on the growth of cancer cell lines were determined following the general procedure used by the National Cancer Institute for *in vitro* anticancer drug study. The procedure uses the protein-binding dye Sulphorhodamine B for the estimation of cell growth[29]. In due course of time, the growth of the cells were counted (95 cells per well in 100 mL medium) in 90 microtitre plates. The study has been conducted keeping the cells for incubation for 40 hrs at 20°C. The experimental set-up of three different wells was conducted where the cells were kept for 36 hrs. This was followed by reacting the cells with 30% cold (5-10°C) TCA. It was left for 2 hrs at 20°C and then washed and dried in air. All the cells were stained with Sulphorhodamine B dye. The dye was dissolved in tris-buffer solution. The plates under study were taken in shaker and kept for 20-30 minutes. The cell growth was calculated using optical density (OD) study and the results were reported in terms of IC_{50} values. Doxorubicin was considered as standard reference.

From the study of IC_{50} values, it has been found that six (6) newly synthesized bis (cycloadducts) showed significant cytotoxicity against human alveolar adenocarcinoma epithelial cells, human cervical cancer cells, human breast adenocarcinoma cells and human breast adenocarcinoma cells respectively. Among all the tested bis cycloadducts **2-13**), **2**, **4** and **7** showed comparatively more potent IC_{50} value against (ATCC No.CCL-180), HeLa derived from human cervical cancer cells (ATCC No. CCL-14), MDA-MB-167 derived from human breast

adenocarcinoma cells (ATCC No. HTB-35) and MCF7 derived from human breast adenocarcinoma cells (ATCC No. HTB-40) as compared to other bis(cycloadducts) (Table 3). Based upon the study, two most potent bis (isoxazolidine derivatives) **2** and **4** were taken for cell cycle analysis. Cell cycle analysis is going on at present.

Conclusion

Mechanochemical procedure (ball-milling) in a solid phase has been reported in the synthesis of variety of bisnitrones and simultaneous double cycloaddition reactions with excellent yields in a minimum time frame following environment friendly green protocol. We believe this simple, cost efficient and time saving methodology will be adopted by many researchers in synthetic organic chemistry. Finally the reported molecules are found to possess promising anticancer activities and thereby creating interest to synthesize many more bis cycloadducts in near future.

Supplementary Information

Supplementary information is available in the website http://nopr.niscpr.res.in/handle/123456789/58776.

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