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Synthesis and screening for acetylcholinesterase inhibitor activity of some novel 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-ones: Derivatives of irbesartan key intermediate

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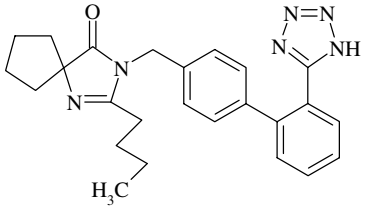
Abstract—The association of bioactive nucleus with other pharmacological agents is hoped to improve the efficacy of the treatment by combining the effects of different pharmacological mechanisms of action. Keeping this in view, a series of 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-one derivatives have been synthesized by interaction of 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-one with different bioactive aralkyl halides in presence of powdered potassium carbonate by two different methods viz., conventional and microwave irradiation. The yields under conventional and microwave irradiation methods were in the range of 60–65% and 80–90%, respectively. The structure elucidation of the new compounds has been carried out with the help of elemental analysis and spectral data. All the synthesized compounds have been screened for their efficacy as acetylcholinesterase (AChE) inhibitor. AChE inhibitory activity study was carried out by using Ellman colorimetric assay with neostigmine as a reference standard against targets from different species, such as pure electric eel AChE, human serum AChE, and rat brain AChE. Among the compounds synthesized, compounds **5a, 5b, 5j** showed good inhibition against AChE.

1. Introduction

The rapid synthesis of a diverse range of libraries of small organic molecules for biochemical uses and drug discovery has become an active sphere of research.[1,2] Due to its high biological profile 2-butyl-1,3-diaza-spiro-[4,4]non-1-en-4-one **3** may be considered as an attractive template among small molecule libraries. Irbesartan (SR47436, BMS-186295), chemically designated as 2-butyl-3-[[29-(1*H*-tetrazole-5-yl)[1,19-biphenyl]-4-yl]methyl]-1,3-diaza-spiro[4,4]non-1-en-4-one **6**, is a potent, long-acting angiotensin II (AII)1 receptor antagonist, with a high selectivity for the AT1 subtype.[3] The chemical structure of irbesartan is shown in Fig. 1. Irbesartan and other AII receptor antagonists have a potentiality to be advantageous in terms of safety and tolerability over earlier classes of drugs for the treatment of hypertension, diabetic nephropathy, and heart failure.[4–6]



(6)

Figure 1.

Alzheimer's disease (AD) is a neurodegenerative disease characterized by a low concentration of acetylcholine (ACh) in the hippocampus and cortex.[7] Inhibition of AChE, an enzyme responsible for the metabolic breakdown of ACh, is considered appropriate for improving AD.[8,9] Currently available AChE inhibitors can treat only a mild to moderate levels of AD disease.[10] There is still a need to develop more efficient drugs for AD. Spiro compounds represent an important class of

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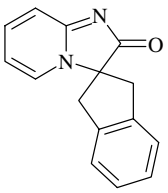


ZSET1446

(7)

Figure 2.

naturally occurring substances characterized by their highly pronounced biological properties.[11–13] The asymmetric characteristic of the molecule resulting from the chiral spiro carbon is one of the important criteria for the biological activities.[14] Of late, the effects of azaindolizinone derivative ZSET1446, spiro[imidazo[1,2-*a*]pyridine-3,2-indon]-2(3*H*)-one **7**, have been assessed in rats with learning deficits induced by $A\beta_{1\text{-}40}$ or scopolamine suggesting that ZSET1446 (Fig. 2) may be a potential candidate for being developed as a therapeutic agent to treat cognitive impairment associated with conditions such as Alzheimer's disease.[15] Title compounds have been taken from these observations to screen their AChE inhibitory activity.

2. Results and discussion

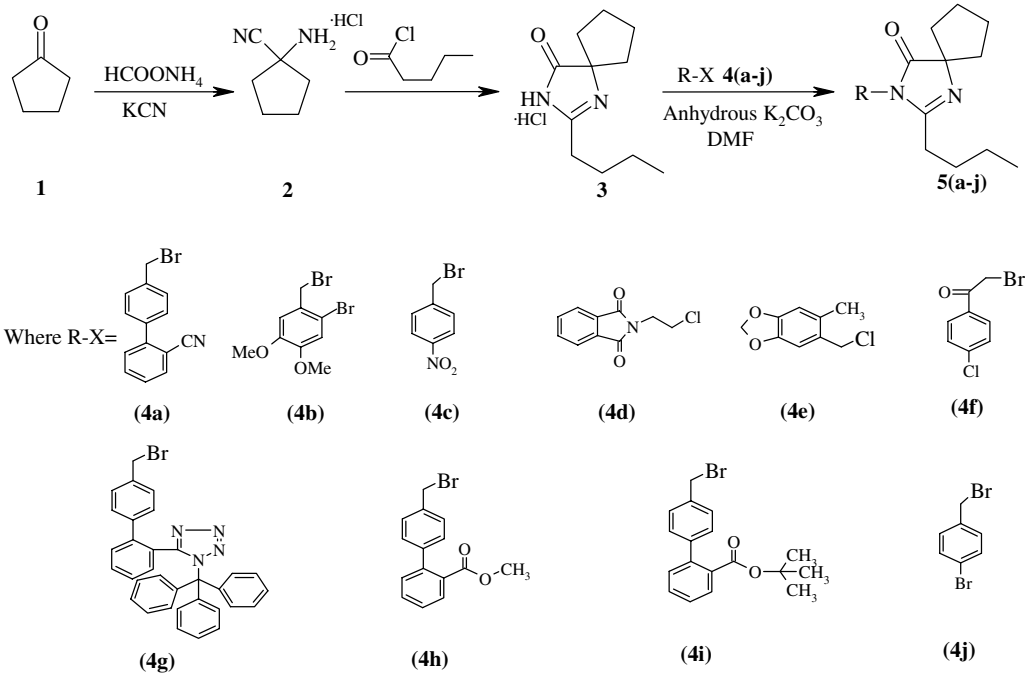
2.1. Chemistry

The bioactive intermediate **3** was synthesized in accordance with the reported procedure.[16] The synthesis of the title compounds was done by condensing different bioactive aralkyl halides with 2-butyl-1,3-diaza-spiro[4-4]non-1-en-4-one **3** in presence of powdered potassium carbonate in DMF (Scheme 1). The compounds **5(a–j)** were synthesized in two different ways viz., conventional and microwave irradiation. Compared to conventional method, microwave heating offers more advantages such as reduced reaction time (65–75 s), low cost, simplicity in processing, reduced pollution and high yield. The yields under conventional and microwave irradiation methods were in the range of 60–65% and 80–90%, respectively. The synthesized compounds were characterized by the spectral (IR, ^{1}H NMR) and elemental analyzes.

IR spectrum of the compound **2** showed bands at 3340 and 2224 cm^{-1} due to $-NH_2$ and CN groups, respectively. ^{1}H NMR of **2** showed doublet at δ 2.4 for two protons due to $-NH_2$ group.

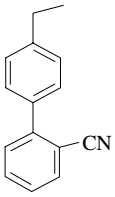
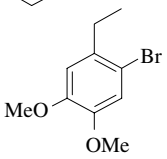
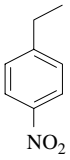
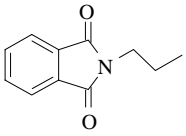
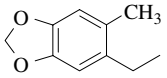
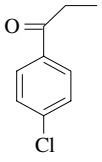
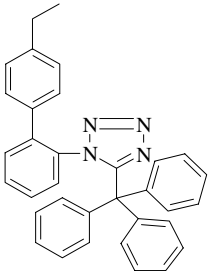
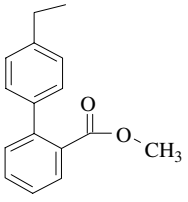
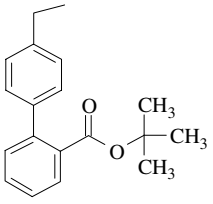
IR spectrum of compound **3** showed bands at 1710 cm^{-1} due to carbonyl group. The band at 2224 cm^{-1} disappeared and the new band appeared at 1480 cm^{-1} due to C=N stretching. ^{1}H NMR of **3** showed singlet at δ 7.8 for 1 proton due to –NH and showed peaks for newly formed ring in the expected region.

IR spectrum of all the compounds **5(a–j)** showed bands at 1730–1740 cm^{-1} due to carbonyl group. Compound **5a** showed the band at 2224 cm^{-1} due to CN group of biphenyl ring. The band at 3320 cm^{-1} disappeared. ^{1}H NMR of all the compounds **5(a–j)** showed absence of peak at δ 7.8 for 1 proton due to –NH group and a new singlet was observed at δ 4.3–4.4 for two protons indicating the formation of the product. All other protons are observed in the expected region. The formation of the product was further confirmed by correct elemental analyzes.



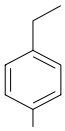
Scheme 1.

Table 1. Reaction condition and physical data of 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-one derivatives **5(a–j)**

Compound	R	Reaction time		Yield (%)		Mp (°C)
		Conventional (h)	MWirr. (S)	Conventional	MWirr.	
5a		6	75	65	90	126
5b		5	65	60	87	134
5c		6	75	65	84	106
5d		6	65	62	83	127
5e		6	65	65	85	128
5f		5	70	60	83	122
5g		5	70	63	90	109
5h		5	65	63	85	118
5i		6	65	65	88	136

(*continued on next page*)

Table 1 (*continued*)

Compound	R	Reaction time		Yield (%)		Mp (°C)
		Conventional (h)	MWirr. (S)	Conventional	MWirr.	
5j		5	70	65	84	121

2.2. Pharmacology

Screening of cholinesterase inhibition was done by determining the IC_{50} value, which is the diazaspiro (ligand) concentration that causes 50 percent inhibition of the cholinesterase activity measured with a given substrate at a specified substrate concentration. The IC_{50} values were determined for inhibition of human serum, for rat brain homogenate, for electric Eel AChE, measured with 1.0 mM acetylthiocholine (ATCh) as substrate, at 37 °C and pH 7.4.

The inhibitory studies of the newly synthesized compounds against AChE using different sources such as rat brain homogenate AChE, human serum AChE, and electric eel AChE are shown in Figures 3–5, respectively. Activities of the synthesized compounds were compared with the inhibitory activity shown by the known standard inhibitor neostigmine. The effects of different or versatile aromatic substituents are explored for their inhibitory activity against AChE.

Among the molecules screened for the AChE inhibitory activity, the compound **5j** (IC_{50} = 70, 70 and 67.5 nM) with bromo group at the 4th position shows highest AChE inhibitory activity. The compounds **5b** and **5a** with bromo group at the 2nd position and cyano group on the biphenyl ring, respectively, are also effective in blocking the AChE enzyme activity (IC_{50} = 77.5, 82.5, 80.0; 90.0, 82.5, 90.0 nM, respectively) (Table 2). But the compounds **5(g–i)** with biphenyl ring with bulky substituent did not show any inhibitory activity. Smaller the substituent, the more favorable activity at the biphenyl methyl site of the molecule **(5a)**. The compounds **5c** and **5f** show moderate activity. This is probably due to the presence of electron withdrawing nitro group **(5c)**

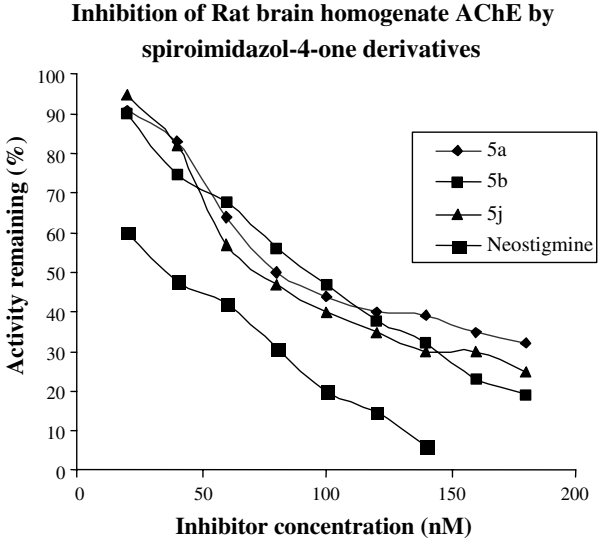


Figure 3.

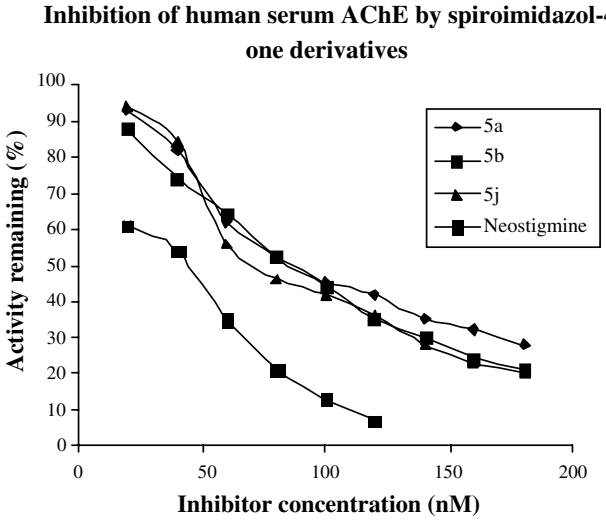


Figure 4.

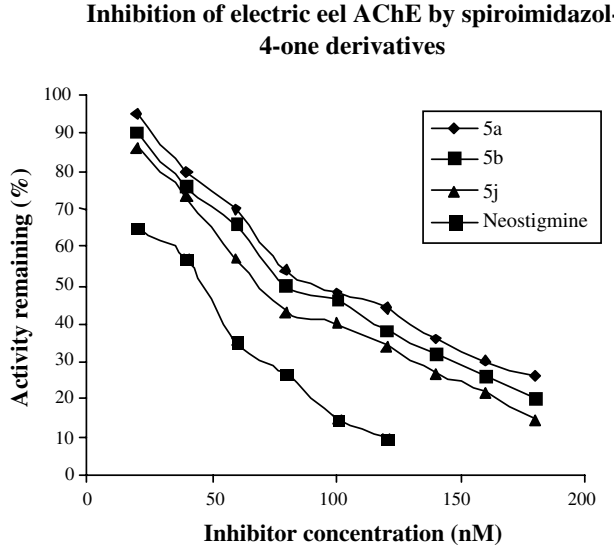


Figure 5.

Table 2. Comparative inhibitory activities of spiroimidazol-4-one derivatives **5(a–j)** against AChE from different sources

Compound	Rat brain homogenate IC_{50} (nM)	Human serum IC_{50} (nM)	Electric eel IC_{50} (nM)
5a	90.0 ± 5	82.5 ± 5	90.0 ± 6
5b	77.5 ± 3	82.5 ± 5	80.0 ± 5
5c	133.6 ± 6	135.4 ± 6	149.2 ± 7
5d	233.8 ± 15	230.8 ± 16	250.1 ± 15
5e	216.2 ± 12	213.5 ± 13	212.4 ± 13
5f	280.4 ± 19	286.4 ± 19	279.6 ± 19
5g	356.5 ± 24	383.5 ± 25	373.2 ± 24
5h	243.2 ± 17	240.6 ± 17	235.7 ± 18
5i	270.6 ± 18	268.8 ± 17	276.8 ± 17
5j	70.0 ± 3	70.0 ± 3	67.5 ± 3
Neostigmine	35.0 ± 2	45.0 ± 4	47.5 ± 5

and chlorine atom at 4th position of the substituent and also because of the ester spacer at the 1st position of the substituent **R**. Compounds **5d** and **5e** having fused rings did not show good inhibitory activity.

It may be observed from the SAR studies that, the spiroimidazol-4-one ring containing single or non-fused aromatic rings (**5j**, **5b**, and **5a**) show a better activity than the fused aromatic rings (**5d** and **5e**). The order of potency is **5j > 5b > 5a**. It may be concluded that, for an effective binding and blocking of the AChE activity, the molecule requires to bind with the peripheral site and the active site of the enzyme and it may be possible that the spiroimidazol-4-one binds at the active site gorge and the different substituents bind to peripheral site separated by the spacer such as methylene or acyl or ester groups. Furthermore, the bulky *n*-butyl chain present on second position of the spiroimidazol-4-one enhances the hydrophobicity which may help in binding to the active site of the enzyme. Therefore, it may be summarized that, the substitution of other smaller rings with smaller electron withdrawing groups on spiroimidazol-4-one basic nucleus needs to be studied for better AChE inhibitory activity. The structures of the potent AChE inhibitor are shown in Figure 6.

3. Conclusions

In conclusion it may be said that, we have succeeded in synthesizing the novel 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-one derivatives **5(a–j)** under both conventional and microwave irradiation technique (solution phase). It is thus concluded that under microwave heating, the products **5(a–j)** were conveniently and efficiently prepared in good yields, typically in the range of 80–90%. The AChE inhibitory activity data reveal that, the compounds **5j**, **5b**, and **5a** are active site-directed irreversible inhibitors of AChE activity. These results could be useful in designing new AChE inhibitors, resulting in greater selectivity as well as an increase in new inhibitor potency.

4. Experimental

The melting points were determined on SELACO-650 hot stage apparatus and remain uncorrected. IR (KBr) spectra were recorded on a Jasco FT/IR-4100 Fourier transform infrared spectrometer, ^{1}H NMR were recorded on Shimadzu AMX 400 spectrometer by using $CDCl_3$ as solvent and TMS as an internal standard (Chemical shift in ppm). Elemental analyzes were performed on a vario-EL instrument. Thin layer chromatography (TLC) was conducted on 0.25 mm silica gel plates ($60F_{254}$, Merck). Visualization was made with ultraviolet light. All extracted solvents were dried over anhydrous Na_2SO_4 and evaporated with a BUCHI rotary evaporator. Reagents were obtained commercially and used as they were received.

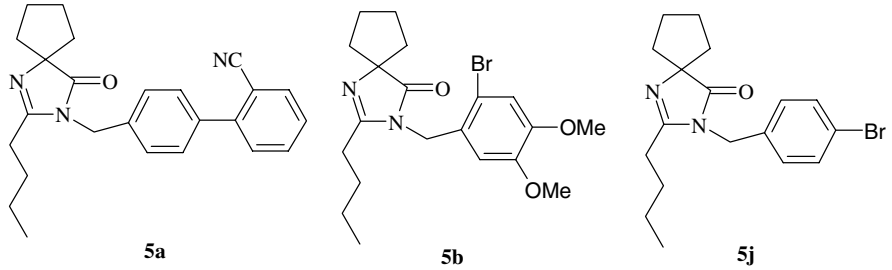
4.1. Synthesis of 1-amino-1-cyano-cyclopentane hydrochloride (2)

A mixture of cyclopentanone (20 g, 238 mmol) and ammonium formate (15 g, 238 mmol) was stirred in 125 ml water to obtain a clear solution. Potassium cyanide (15.5 g, 238 mmol) was spooned into the solution over 5 min with a little heat evolution; an oily phase began to separate. The reaction mixture was stirred at ambient temperature for about 24 h. The upper layer was separated and it was diluted to 75 ml with toluene and then dried over anhydrous sodium sulfate. The toluene mixture was saturated with dry hydrogen chloride gas, giving separation of solid (a precipitate). The solid was filtered, washed with toluene and hexane, and dried at 50 °C under vacuum. Yield 68%; mp: 156–158 °C.

IR ν_{max} (KBr): 3340, 2985, 2224, 1480 cm^{-1}.

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.5–1.95 (m, 8H, cyclopentyl), 2.4 (d, 2H, $-NH_2$).

Anal. Calcd for $C_6H_{10}N_2$: C, 65.42; H, 9.15; N, 25.43. Found: C, 65.43; H, 9.18; N, 25.42%.



5a **5b** **5j**

Figure 6. Structures of potent AChE inhibitor.

4.2. Synthesis of 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-one monohydrochloride (3)

To the solution of 1-amino-1-cyanocyclopentane (15 g, 136 mmol) in 150 ml of toluene triethylamine (20.6 g, 204 mmol; 1.5 equiv) was added and valeroyl chloride (19.7 g, 163 mmol; 1.2 equiv) was added to this solution dropwise, under cooling, at 20 °C. After 10–15 min the temperature was raised to 80 °C and the mixture was stirred at that temperature for about 2 h. The reaction mixture was then cooled to room temperature and was extracted consecutively with 50 ml of water, 40 ml of 2% hydrochloric acid solution, and 40 ml of water. The toluene solution was evaporated under vacuo. To the residue a solution of potassium hydroxide (3.8 g, 68 mmol; 0.5 equiv) in 50 ml of methanol was added at first and then under cooling a 30 ml of 30% hydrogen peroxide solution. After the addition of hydrogen peroxide, the temperature was maintained at 50 °C for about 30 min. The solution was cooled to room temperature and added potassium hydroxide (15.3 g, 272 mmol; 2 equiv) solution. The mixture was heated under reflux condition for about 2 h. The reaction was then frozen by adding 30 g of ammonium chloride and methanol was distilled out. The reaction mixture was then extracted with ethyl acetate (50× 3 ml). The combined organic phase was evaporated in vacuo, the residue was dissolved in 5-fold amount of acetone, the resulting solution was filtered, its pH was adjusted to 1–2 by the addition of concd hydrochloric acid solution. The suspension thus obtained was stirred for 1 h, cooled to 0 °C. The crystals were filtered off, washed with cold acetone, and dried to obtain the product **3**. Yield 55%; mp: 68 °C

IR ν_{max} (KBr): 2995, 1710, 1480, 1455, 1365 cm^{-1}.

^1H NMR ($CDCl_3$, 400 MHz) δ: 0.88–0.92 (t, 3H, $–CH_3$), 1.2–1.4 (m, 6H, $–CH_2–CH_2–CH_2$), 1.5–1.95 (m, 8H, cyclopentyl), 7.8–8.2 (s, 1H, –NH).

Anal. Calcd for $C_{11}H_{18}N_2O$: C, 68.01; H, 9.34; N, 14.42. Found: C, 68.03; H, 9.35; N, 14.45%.

4.3. General procedures for the synthesis of 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-one derivatives 5(a–j)

[a] Conventional method. A mixture of 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-one **3** (1 equiv), aralkyl halides **4(a–j)** (1.2 equiv), and powdered potassium carbonate (1.2 equiv) in *N*,*N*-dimethylformamide (10 ml) was stirred at room temperature for about 6–8 h. The reaction was monitored by thin layer chromatography. After the completion of the reaction, deionized water was added to the reaction mixture and extracted with ethyl acetate (3× 10 ml). The combined organic layer was dried over anhydrous Na_2SO_4. The crude products **5(a–j)** obtained on evaporation of the solvent under reduced pressure were purified by column chromatography using hexane and ethyl acetate as eluents.

[b] Microwave irradiation method. A 25 ml conical flask charged with 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-one **3** (1 g, 5.15 mmol), different aralkyl halides **4(a–j)** (1.2 equiv), and DMF (10 ml) was irradiated in the microwave oven at 20% power level (60 W) for 65–75 s. After completion of the reaction (tlc), 10 equivalent of water was added to the cooled (rt) contents of the flask. Using the above workup procedure isolated the pure products. The reaction condition and physical data of the synthesized compounds are depicted in Table 1.

4.3.1. Synthesis of 4′-(2-butyl-4-oxo-1,3-diaza-spiro[4,4]-non-1-en-3-ylmethyl)-biphe-nyl-2-carbonitrile (5a). The product **5a** was obtained from 2-butyl-1,3-diaza-spiro-[4,4]non-1-en-4-one **3** (1 g, 5.15 mmol) and 4′-bromomethyl-biphenyl-2-carbonitrile **4a** (1.7 g, 6.18 mmol).

IR ν_{max} (KBr): 3095, 2224, 1735, 1574, 1480 cm^{-1}.

^1H NMR ($CDCl_3$ 400 MHz) δ: 0.88–0.92 (t, 3H, $–CH_3$), 1.2–1.4 (m, 6H, $–CH_2–CH_2–CH_2–$), 1.51–1. 81 (m, 8H, –cyclopentane), 4.34 (s, 2H, $–CH_2–$), 7.15–8.00 (m, 8H, Ar-H).

Anal. Calcd for $C_{25}H_{27}N_3O$: C, 77.89; H, 7.06; N, 10.90. Found: C, 77.91; H, 7.04; N, 10.89%.

4.3.2. Synthesis of 3-(2-bromo-4,5-dimethoxybenzyl)-2-butyl-1,3-diazaspiro[4,4]non-1-en-4-one (5b). The product **5b** was obtained from 2-butyl-1,3-diaza-spiro[4,4]-non-1-en-4-one **3** (1 g, 5.15 mmol) and 1-bromo-2-bromomethyl-4,5-dimethoxy-benzene **4b** (1.9 g, 6.17 mmol).

IR ν_{max} (KBr): 3105, 2940, 1739, 1572, 1482, 1455, 1367 cm^{-1}.

^1H NMR ($CDCl_3$ 400 MHz) δ: 0.88–0.92 (t, 3H, $–CH_3$), 1.2–1.4 (m, 6H, $–CH_2–CH_2–CH_2–$), 1.51–1. 81 (m, 8H, –cyclopentane), 3.81 (s, 6H–OCH_3), 4.34 (s, 2H, $–CH_2–$), 6.46 (s,1H, Ar-H), 6.75 (s, 1H, Ar-H).

Anal. Calcd for $C_{20}H_{27}BrN_2O_3$: C, 56.74; H, 6.43; N, 6.62. Found: C, 56.73; H, 6.43; N, 6.64%.

4.3.3. Synthesis of 2-butyl-3-(4-nitrobenzyl)-1,3-diazaspiro[4,4]non-1-en-4-one (5c). The product **5c** was obtained from 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-one **3** (1 g, 5.15 mmol) and 1-bromomethyl-4-nitro-benzene **4c** (1.3 g, 6.17 mmol).

IR ν_{max} (KBr): 3100, 2935, 1732, 1565, 1480, 1445, 1355 cm^{-1}.

^1H NMR ($CDCl_3$ 400 MHz) δ: 0.88–0.92 (t, 3H, $–CH_3$), 1.2–1.4 (m, 6H, $–CH_2–CH_2–CH_2–$), 1.51–1.81(m, 8H, –cyclopentane), 4.34 (s, 2H, $–CH_2–$), 7.15–7.18 (d, 4H, Ar-H).

Anal. Calcd for $C_{18}H_{25}N_3O_3$: C, 65.24; H, 7.60; N, 12.68. Found: C, 65.24; H, 7.61; N, 12.68%.

4.3.4. Synthesis of 2-[2-(2-butyl-4-oxo-1,3-diazaspiro[4,4]non-1-en-3-yl)ethyl]isoin-dole-1,3-dione (5d). The product **5d** was obtained from 2-butyl-1,3-diaza-spir-

o[4,4]non-1-en-4-one **3** (1 g, 5.15 mmol) and 2-(2-chloroethyl)-isoindole-1,3-dione **4d** (1.2 g, 6.18 mmol).

IR ν_{max} (KBr): 3099, 2932, 1734, 1578, 1495, 1465, 1365 cm^{-1}.

^1H NMR ($CDCl_3$ 400 MHz) δ: 0.88–0.92 (t, 3H, $-CH_3$), 1.2–1.4 (m, 6H, $-CH_2-CH_2-CH_2-$), 1.51–1.81 (m, 8H, –cyclopentane), 3.65–3.85 (t, 4H, $-CH_2-CH_2-$), 7.72–8.35 (m, 4H, Ar-H).

Anal. Calcd for $C_{21}H_{25}N_3O_3$: C, 68.64; H, 6.86; N, 11.44. Found: C, 68.63; H, 6.87; N, 11.45%.

4.3.5. Synthesis of 2-butyl-3-(6-methylbenzo[1,3]dioxole-5-ylmethyl)-1,3-diazaspiro [4,4]non-1-en-4-one (5e). The product **5e** was obtained from 2-butyl-1,3-diaza-spiro[4,4]-non-1-en-4-one **3** (1 g, 5.15 mmol) and 5-chloromethyl-6-methyl-benzo[1,3]dioxole **4e** (1.4 g, 6.18 mmol).

IR ν_{max} (KBr): 3095, 2930, 1735, 1574, 1480, 1465, 1369 cm^{-1}.

^1H NMR ($CDCl_3$ 400 MHz) δ: 0.88–0.92 (t, 3H, $-CH_3$), 1.2–1.4 (m, 6H, $-CH_2-CH_2-CH_2-$), 1.51-1.81(m, 8H, –cyclopentane), 4.34 (s, 2H, $-CH_2-$), 5.8 (s, 2H, $-CH_2$-), 6.46 (s, 3H, Ar-H), 5.8 (s, 3H, $-CH_3$).

Anal. Calcd for $C_{20}H_{26}N_2O_3$: C, 70.15; H, 7.65; N, 8.18. Found: C, 70.13; H, 7.65; N, 8.19%.

4.3.6. Synthesis of 2-butyl-3-[2-(4-chlorophenyl)-2-oxoethyl]-1,3-diazaspiro[4,4]non-1-en-4-one (5f). The product 5**f** was obtained from 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-one **3** (1 g, 5.15 mmol) and 2-Bromo-1-(4-chlorophenyl)ethanone **4f** (1.4 g, 6.18 mmol).

IR ν_{max} (KBr): 3115, 2940, 1743, 1572, 1488, 1455, 1345 cm^{-1}.

^1H NMR ($CDCl_3$ 400 MHz) δ: 0.88–0.92 (t, 3H, $-CH_3$), 1.2–1.4 (m, 6H, $-CH_2-CH_2-CH_2-$), 1.51–1.81 (m, 8H, –cyclopentane), 4.34 (s, 2H, $-CH_2-$), 7.65 (dd, 2H, Ar-H), 7.88 (dd, 2H, Ar-H).

Anal. Calcd for $C_{19}H_{23}ClN_2O_2$: C, 65.79; H, 6.68; N, 8.08. Found: C, 65.78; H, 6.69; N, 8.10%.

4.3.7. Synthesis of 2-butyl-3-[2′-(1-trityl-tetrazol-5-yl)biphenyl-4-ylmethyl]-1,3-diaza spiro[4,4]non-1-en-4-one (5g). The product **5g** was obtained from 2-butyl-1, 3-diaza-spiro[4,4]non-1-en-4-one **3** (1 g, 5.15 mmol) and 5-(4′-bromomethyl-biphenyl-2-yl)-1-trityl-1*H*-tetrazole **4g** (3.4 g, 6.18 mmol).

IR ν_{max} (KBr): 3114, 2945, 1740, 1570, 1486, 1451, 1340 cm^{-1}.

^1H NMR ($CDCl_3$ 400 MHz) δ: 0.88–0.92 (t, 3H, $-CH_3$), 1.2–1.4 (m, 6H, $-CH_2-CH_2-CH_2-$), 1.51–1.81(m, 8H, –cyclopentane), 4.34 (s, 2H, $-CH_2-$), 6.79–8.05 (m, 24H, Ar-H).

Anal. Calcd for $C_{44}H_{42}N_6O$: C, 78.78; H, 6.31; N, 12.53. Found: C, 78.79; H, 6.32, N, 12.52%.

4.3.8. Synthesis of 4′-(2-butyl-4-oxo-1,3-diazaspiro[4,4]non-1-en-3-ylmethyl)-biphe-nyl-2-carboxylic acid methyl ester (5h). The product **5h** was obtained from 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-one **3** (1 g, 5.15mmol) and 4′-bromomethyl-biphenyl-2-carboxylic acid methyl ester **4h** (1.9 g, 6.18 mmol).

IR ν_{max} (KBr): 3105, 2937, 1740, 1576, 1467, 1465, 1354 cm^{-1}.

^1H NMR ($CDCl_3$ 400 MHz) δ: 0.88–0.92 (t, 3H, $-CH_3$), 1.2–1.4 (m, 6H, $-CH_2-CH_2-CH_2-$), 1.51–1.81(m, 8H, –cyclopentane), 3.92–3.93 (s, 3H, $-OCH_3$), 4.34 (s, 2H, $-CH_2$-), 7.11–8.00 (m, 8H, Ar-H).

Anal. Calcd for $C_{26}H_{30}N_2O_3$: C, 74.61; H, 7.22; N, 6.69. Found: C, 74.61; H, 7.23; N, 6.69%.

4.3.9. Synthesis of 4′-(2-butyl-4-oxo-1,3-diazaspiro[4,4]-non-1-en-3-ylmethyl)-biphenyl-2-carboxylic acid tert-butyl ester (5i). The product **5i** was obtained from 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-one **3** (1 g, 5.15 mmol) and 4′-bromomethyl-biphenyl-2-carboxylic acid *tert*-butyl ester **4i** (2.1 g, 6.17 mmol).

IR ν_{max} (KBr): 3017, 2945, 1740, 1578, 1493 cm^{-1}.

^1H NMR ($CDCl_3$ 400 MHz) δ: 0.88–0.92 (t, 3H, $-CH_3$), 0.97–1.00 (s, 9H, $-C(CH_3)_3$), 1.2–1.4 (m, 6H, $-CH_2-CH_2-CH_2-$), 1.51–1.81(m, 8H, –cyclopentane), 4.34 (s, 2H, $-CH_2-$), 7.12–7.89 (m, 8H, Ar-H).

Anal. Calcd for $C_{29}H_{36}N_2O_3$: C, 75.62; H, 7.88; N, 6.08. Found: C, 75.61; H, 7.87; N, 6.09%.

4.3.10. Synthesis of 3-(4-bromobenzyl)-2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-one (5j). The product **5j** was obtained from 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-one **3** (1 g, 5.15 mmol) and 1-bromo-4-bromomethyl-benzene **4j** (1.5 g, 6.18 mmol).

IR ν_{max} (KBr): 3098, 2941, 1740, 1579, 1478, 1462, 1345 cm^{-1}.

^1H NMR ($CDCl_3$ 400 MHz) δ: 0.88–0.92 (t, 3H, $-CH_3$), 1.2–1.4 (m, 6H, $-CH_2-CH_2-CH_2-$), 1.51–1.81 (m, 8H, –cyclopentane), 4.34 (s, 2H, $-CH_2-$), 7.25 (dd, 2H, Ar-H), 7.46 (dd, 2H, Ar-H).

Anal. Calcd for $C_{18}H_{23}BrN_2O$: C, 59.51; H, 6.38; N, 7.71. Found: C, 59.51; H, 6.39; N, 7.70%.

5. Biology: in vitro acetylcholinesterase assay

The cholinesterase assay method of Ellman et al.[17] was used to determine the in vitro cholinesterase activity. The activity was measured by increase in absorbance at 412 nm due to the yellow color produced from the reaction of acetylthiocholine iodide with the dithiobisnitrobenzoate (DTNB) ion. AChE was obtained from the brain of Wistar rats by homogenizing under a Teflon blender for 10 min in 0.1 M KH_2PO_4 buffer,

pH 8. A stock solution of the enzyme in 0.1 M KH_2PO_4 buffer (pH 8) was kept frozen. For each assay 300 μg of enzyme was used. Acetylthiocholine iodide was prepared daily using 0.1 M KH_2PO_4 buffer (pH 7). A 0.01 M solution of DTNB was prepared in 0.1 M KH_2PO_4 buffer (pH 7). Crude human AChE was prepared by mixing 9 ml of fresh blood (collected from healthy volunteers by vein puncture) with 1 ml of 3.8 % (w/v) trisodium citrate and centrifuging at 3000 rpm at 0 °C for 20 min. The supernatant was used as a source of AChE. Electric eel AChE was obtained from Sigma Laboratory and similar procedure was employed for the assay as that of rat brain AChE.

5.1. Experimental conditions and kinetics

Enzyme activity was measured using a Shimadzu Spectrophotometer. The assay medium contained phosphate buffer, pH 8.0 (2.6 ml), DTNB (0.1 ml), 5 μl of enzyme, 20 μl of 0.075 M substrate. The activity was determined by measuring the increase in absorbance at 412 nm at 1 min intervals for 10 min at 37 °C. In dose-dependent inhibition studies, the substrate was added to the assay medium containing enzyme, buffer, and DTNB with inhibitor after 10 min of incubation time. Calculations were performed according to the method of the equation in Ellman et al.[17] All experiments were carried out in duplicate and the mean values are reported here. The relative activity is expressed as percentage ratio of enzyme activity in the absence of inhibitor.

5.1.1. Protein estimation. Protein content was determined by the Lowry method[18] using bovine serum albumin as standard.

5.1.2. IC_{50} determination. The inhibition by spiroimidazol-4-one derivatives was studied in the presence of different concentrations of compounds and the percentage inhibition of enzyme activity was calculated. The results obtained were analyzed with the values obtained in comparison to that of neostigmine.

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