Arch Pharm Res Vol 35, No 8, 1369-1377, 2012
DOI 10.1007/s12272-012-0806-3

Archives of Pharmacal Research
www.springer.com/12272

Design and Synthesis of Novel 1,4-Benzodiazepine Derivatives and Their Biological Evaluation as Cholinesterase Inhibitors

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(Received October 17, 2011/Revised November 30, 2011/Accepted December 6, 2011)

A new series of 1,4-benzodiazepine-2,5-dione structurally related to cyclopenin has been synthesized. The new compounds were assayed in *vivo* and in *vitro* for their ability to inhibit acetylcholinesterase enzyme and were found to have potent reversible anticholinesterase activity when tested in *vitro* for isolated frog rectus abdominis and guinea pig ileum in addition to increasing brain cholinesterase level in rats when percentage inhibition were tested in *vivo*, moreover compounds **5a**, **5b, 5c** and **5g** were the most active. LD_{50} was performed for these derivatives and they displayed high safety margin.

Key words: 1,4-Benzodiazepine, Acetylcholine, Alzheimer's disease, Cyclopenin, Cyclopenol

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INTRODUCTION

Acetylcholinesterase (Ach E) inhibitors gained much interest in the last few years due to their ability to treat Alzheimer's disease and similar cognitive disorders, conditions characterized by a cholinergic deficiency in the cortex and basal forebrain (Williams et al., 2002). The successful development of these compounds was based on the well- accepted hypothesis that the decline in cognitive and mental functions associated with Alzheimer's disease is related to the loss of cortical cholinergic neurotransmission (Adreani et al., 2001). Inhibition of Acetylcholinesterase prolongs the duration of the neurotransmitter in the junction and produces pharmacologic effects similar to those observed when acetylcholine is administered. Acetylcholinesterase is present throughout the central and peripheral nervous systems of mammals, where it catalyses the hydrolysis of the endogenous ester neurotransmitter acetylcholine, allowing the termination of acetylcholine (ACh) receptor-mediated ion gating at nerve–nerve and neuromuscular junctions (Geissler et al., 2010).

Recent study has shown that AChE inhibitors can be effective over a longer period for that AChE plays an important role in Aβ deposition (a peptide of 39–43 amino acids that appear to be the main constituent of amyloid plaques in the brains of Alzheimer's disease patients) (Zhou et al., 2008).

Recent research revealed that several AChE inhibitors not only facilitate cholinergic transmission, but also interfere with the synthesis, deposition and aggregation of Aβ. Accordingly AChE inhibitors have become leading strategy for the development of anti-Alzheimer's disease agents. Tacrine has been the first FDA-approved Alzheimer's disease treatment agent, although the patient suffers from its dose-limiting side effects (Kuno et al., 1996). Tacrine was followed by other drugs developed for the treatment of Alzheimer's disease, such as physostigmine, Huperzine and rivastigmine (Fig. 1).

1, 4-Benzodiazepines have been known for their excellent anxiolytic, hypnotic, muscle relaxant and anticonvulsant properties; 1, 4-benzodiazepines rank among the most widely used pharmaceuticals in the developed countries (Scholl et al., 1983; Grover et al., 2011). Diazepam, as cholinesterase inhibitor, was found to increase the free and bound acetylcholine levels in the mouse

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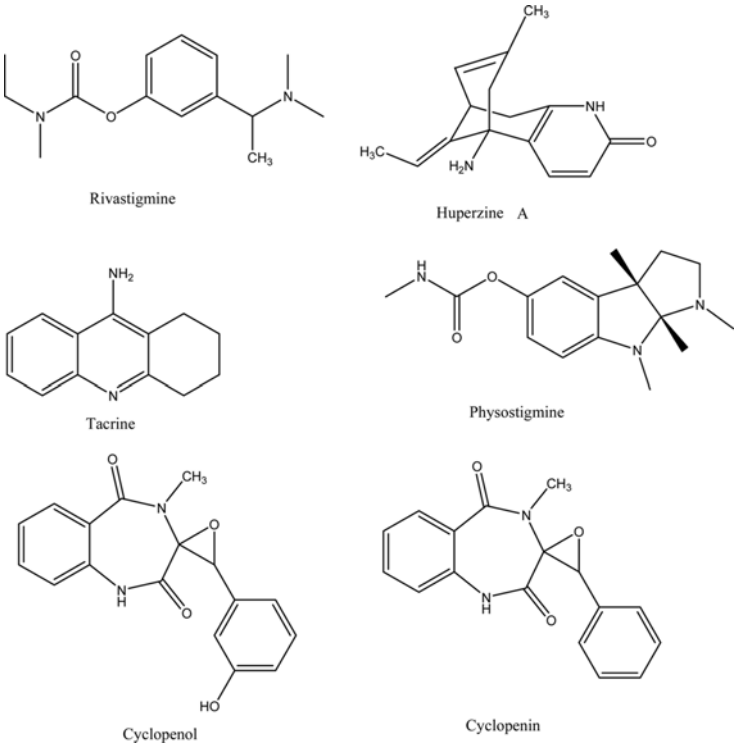


Fig. 1. Structures of some anticholinesterase compounds.

brain (Consolo et al., 1974; Tankopii et al., 1978; Schultz et al., 2012). The biogenic benzodiazepine alkaloids exert various biological effects, indicating that the benzodiazepine nucleus is not a sufficient prerequisite for efficient interaction with the drug receptor but the nature of the substituent that are positioned by the structure of benzodiazepine is of prime importance for the resulting biological activity (Ross, 1990). Cyclopenin is one of the unusual family of benzodiazepine; it was first isolated from *penicillium cyclopium* and *penicillium viridacatum* (Smith et al., 1968). Then, it was synthesized from anthranilic acid and phenylalanine to confirm its structure (Martin et al., 1969; Nover, 1969). In case of cyclopenin and cyclopenol they were found to have cholinesterase inhibitor activity; with selective inhibition against acetylcholinesterase than butyrylcholinesterase (Kuno et al., 1996).

In the present study, new derivatives analogues to cyclopenin were synthesized.

MATERIALS AND METHODS

Chemistry

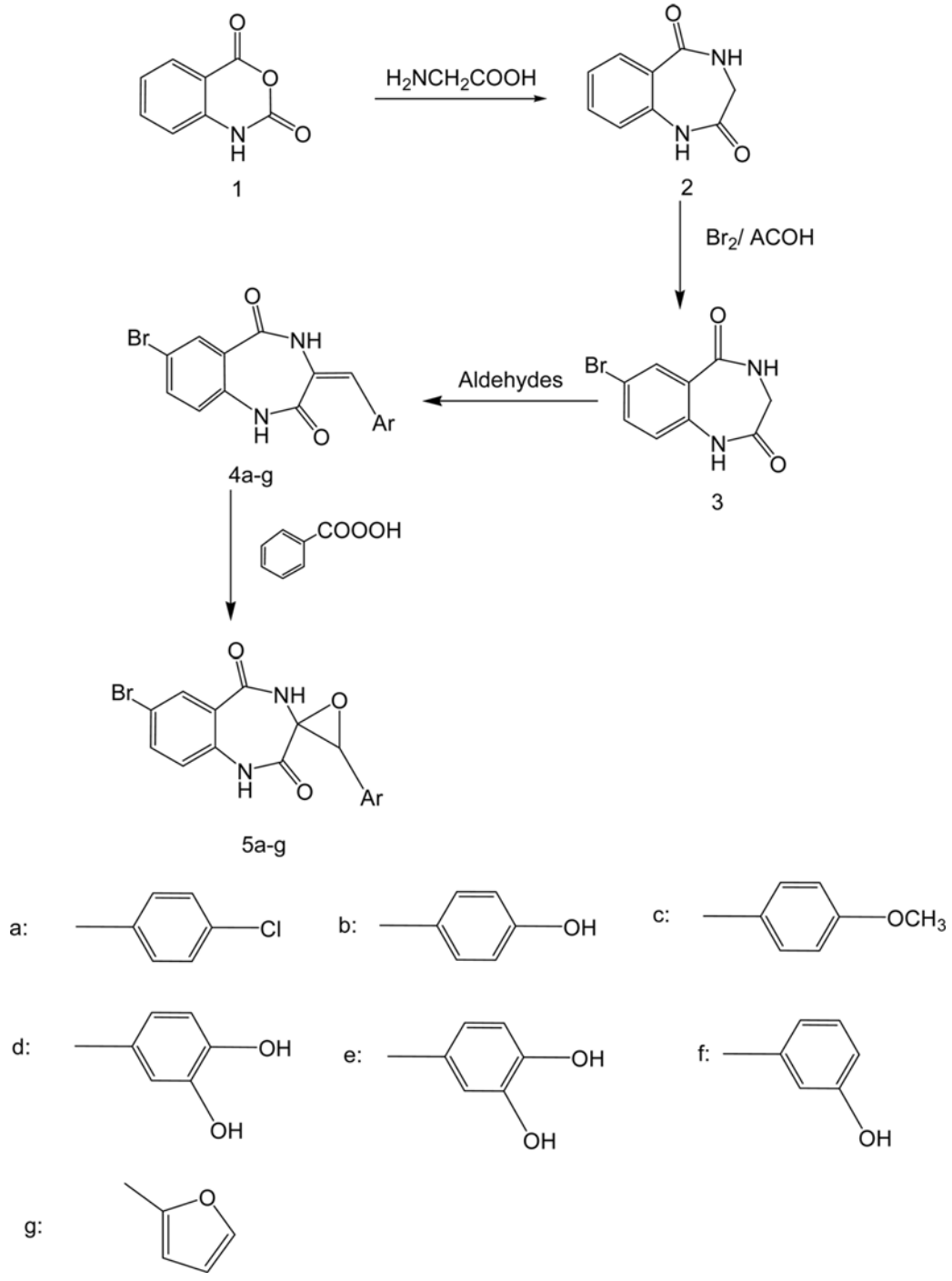
Melting points (mp) were uncorrected and measured in open capillary tubes using griffin apparatus, element analyses were carried out at the micro analytical center Cairo University. Infra Red Spectra were recorded using KBr discs on a Shimadzu 435 spectrometer. Proton Magnetic Resonance measurements were performed on: NMR Varian Gemini 200 MHZ Spectrometer, using TMS as internal standard. Mass Spectra were recorded on Shimadzu Qp-2010 Plus Spectrometer, Micro analytical center, Cairo University. All reactions were monitored by TLC using precoated aluminum sheets silica gel Merck 60 F 254 and were visualized by UV lamp.

General procedure for the preparation of compounds 4a-g

A mixture of **3** (35.5 mmol, 9.05 g), appropriate aldehyde (51.8 mmol), sodium acetate (39 mmol, 3.2 g) and acetic anhydride (10 mL) was heated at 150°C for 3 h. The reaction mixture was then cooled poured on ice-cold water, stirred at room temperature for 1 h. Filtered and the residue dissolved in ethyl acetate (10 mL), the solvent was then evaporated to dryness under reduced pressure. The residue was then triturated with diethyl ether; the formed solid product was dried and crystallized from benzene-ethanol (1:1) mixture.

3-(4-chlorobenzylidine)-7-bromo-3,4-dihydro-1H-benzo[e][1,4]diazepine-2,5-dione 4a

Yield 60%, yellow crystals; mp 240-242°C; IR (KBr) γ cm^{-1}: 3568, 3466, 3049, 2927, 1738, 1684, and 1626. ^{1}H-NMR (DMSO-d_6) δ 10.0 (s, 1H, NH), 9.8 (s, 1H, NH), 7.95 (d, 2H, Ph, J = 9 Hz), 6.91 (d, J = 3 Hz, 2H,



Scheme 1. Synthesis of **5a~g**.

Ph), 7.6 (s, 1H, =CH), 7.58 (s, 1H, Ph), 7.54-7.52 (d, *J* = 7 Hz, 2H, Ph). Anal. Calcd for $C_{16}H_{10}BrClN_2O_2$: C, 50.92; H, 2.65; N, 7.42. Found: C, 50.92; H, 2.22; N, 7.3.

3-(4-hydroxybenzylidene)-7-bromo-3,4-dihydro-1*H*-benzo[e][1,4] diazepine-2,5-dione 4b

Yield 80%, yellow crystals; mp >300ºC; MS *m/z* 358.00, 359.95 (M^+, $M^+ + 2$) IR (KBr) γ cm^{-1}: 3560, 3460, 3211, 2927, 1760, 1684 and 1603. ^{1}H-NMR (DMSO-d_6) δ 9.96 (s, 1H, NH), 9.9 (s, 1H, NH), 7.6 (s, 1H, Ph), 7.53 (d, *J* = 6 Hz, 2H, Ph), 7.33 (s, 1H, =CH), 7.24 (d, *J* = 9 Hz, 2H, Ph), 7.11 (d, *J* = 3 Hz, 2H, Ph), 5.56 (s, 1H, OH). Anal. Calcd for $C_{16}H_{11}BrN_2O_3$: C, 53.48; H, 3.06; N, 7.79. Found: C, 53.50; H, 3.21; N, 8.0.

3-(4-methoxybenzylidene)-7-bromo-3,4-dihydro-1H-benzo[e][1,4] diazepine-2,5-dione 4c
Yield 70%, yellow crystals; mp >300°C; IR (KBr) γ cm^{-1}: 3560, 3460, 3149, 2927, 1762, 1691 and 1599. ^{1}H-NMR (DMSO-d_6) δ 9.97 (s, 1H, NH), 9.8 (s, 1H, NH), 7.97 (d, J = 3 Hz, 2H, Ph), 7.93 (d, J = 7 Hz, 2H, Ph), 7.50 (s, 1H, =CH), 7.54 (s, 1H, Ph), 7.50-7.46 (d, J = 9 Hz, 2H, Ph), 3.38 (s, 3H, OCH_3). Anal. Calcd for $C_{17}H_{13}BrN_2O_3$: C, 54.69; H, 3.48; N, 7.50. Found: C, 55.00; H, 3.69; N, 7.39.

3-(3,4-dihydroxybenzylidene)-7-bromo-3,4-dihydro-1*H*-benzo[e][1,4] diazepine-2,5-dione 4d
Yield 45%, yellow crystals; mp 150-152°C; IR (KBr) γ cm^{-1}: 3560, 3508, 3087, 2993, 1772, 1749 and 1689. ^{1}H-NMR (DMSO-d_6) δ 10.04 (s, 1H, NH), 9.9 (s, 1H, NH), 7.6 (s, 1H, Ph), 7.54 (d, J = 8 Hz, 2H, Ph), 6.55 (s, 1H, =CH), 7.39 (s, 1H, Ph), 7.35 (d, J = 8.2 Hz, 2H, Ph), 7.7 (s, 1H, OH), 7.4 (s, 1H, OH). Anal. Calcd for $C_{16}H_{11}BrN_2O_4$: C, 51.20; H, 2.93; N, 7.46. Found: C, 51.52; H, 2.77; N, 7.49.

3-(4-hydroxy-3-methoxybenzylidene)-7-bromo-3,4-dihydro-1*H*-benzo[e][1,4] diazepine-2,5-dione 4e
Yield 55%, yellow crystals; mp >300°C; IR (KBr) γ cm^{-1}: 3560, 3460, 3211, 2927, 1770, 1691 and 1651. ^{1}H-NMR (DMSO-d_6) δ 9.96 (s, 1H, NH), 9.92 (s, 1H, NH), 7.6 (s, 1H, Ph), 7.53 (d, J = 8.1 Hz, 2H, Ph), 7.37 (s, 1H, =CH), 7.24 (s, 1H, Ph), 7.11 (d, J = 9.2 Hz, 2H, Ph), 5.56 (s, 1H, OH), 3.8 (s, 3H, OCH_3). Anal. Calcd for $C_{17}H_{13}BrN_2O_4$: C, 52.44; H, 3.34; N, 7.197. Found: C, 51.92; H, 3.77; N, 7.18.

3-(3-hydroxybenzylidene)-7-bromo-3,4-dihydro-1*H*-benzo[e][1,4] diazepine-2,5-dione 4f
Yield 45%, yellow crystals; mp 70-72°C; MS m/z 358.1, 360.1 (M^+, M^+ + 2) IR (KBr) γ cm^{-1}: 3560, 3528, 3064, 2980, 1764, 1681 and 1629. ^{1}H-NMR (DMSO-d_6) δ 9.96 (s, 1H, NH), 9.92 (s, 1H, NH), 7.6 (d, J = 4 Hz, 2H, Ph), 7.53 (d, J = 7 Hz, 2H, Ph), 6.5 (s, 1H, =CH), 7.24 (s, 1H, Ph), 7.19-7.16 (d, J = 8.8 Hz, 2H, Ph), 7.4 (s, 1H, OH). Anal. Calcd for $C_{16}H_{11}BrN_2O_3$: C, 53.48; H, 3.06; N, 7.80. Found: C, 53.45; H, 2.96; N, 7.79.

7-bromo-3-(furan-2-yl)methylene)3,4-dihydro-1*H*-benzo[e][1,4] diazepine-2,5-dione 4g
Yield 60%, dark grey crystals; mp 116-118°C; MS m/z 332.9, 334.9 (M^+, M^+ + 2) IR (KBr) γ cm^{-1}: 3555, 3528, 3207, 3145, 2968, 1770, 1683 and 1604. ^{1}H-NMR (DMSO-d_6) δ 9.92 (s, 1H, NH), 9.90 (s, 1H, NH), 8.14 (d, J = 4.2 Hz, 2H, furyl), 8.0 (s, 1H, Ph), 7.7 (s, 1H, Ph), 7.6 (s, 1H, Ph), 7.4 (s, 1H, furyl), 6.9 (s, 1H, =CH). Anal. Calcd for $C_{14}H_9BrN_2O_3$: C, 50.45; H, 2.70; N, 8.41. Found: C, 51.92; H, 2.77; N, 9.13.

General procedure for the preparation of compounds 5a-g

A solution of (1.44 mmol) of compounds **4a-g** and (300 mg, 1.58 mmol) of peroxybenzoic acid in chloroform (10 mL) was allowed to stand at room temperature for 14 days. The solvent was evaporated to dryness under reduced pressure. The residue thus formed was crystallized from diethyl ether.

7-Bromo-3'-(4-chlorophenyl)spiro[1,4-benzodiazepine-3,2'-oxirane]-2,5(3H,4H)-dione 5a
Yield 60%, pale yellow crystals; mp 216°C; IR (KBr) γ cm^{-1}: 3560, 3460, 3049, 2920,1764 and 1684. ^{1}H-NMR (DMSO-d_6) δ 9.96 (s, 1H, NH), 9.8 (s, 1H, NH), 7.6 (s, 1H, Ph), 7.53 (d, J = 7 Hz, 2H, Ph), 7.24 (d, J = 6 Hz, 2H, Ph), 7.19 (d, J = 4.1 Hz, 2H, Ph), 4.5 (s, 1H, CH). Anal. Calcd for $C_{16}H_{10}BrClN_2O_3$: C, 48.58; H, 2.54; N, 7.12. Found: C, 48.60; H, 2.30; N, 7.02.

7-Bromo-3'-(4-hydroxyphenyl)spiro[1,4-benzodiazepine-3,2'-oxirane]-2,5(3H,4H)-dione 5b
Yield 85%, pale yellow crystals; mp >300°C; IR (KBr) γ cm^{-1}: 3560, 3540, 3164, 2980, 1762 and 1687. ^{1}H-NMR (DMSO-d_6) δ 9.96 (s, 1H, NH), 9.92 (s, 1H, NH), 7.6 (s, 1H, Ph), 7.53 (d, J = 5.1 Hz, 2H, Ph), 7.240 (d, J = 6.2 Hz, 2H, Ph), 7.16 (d, J = 7 Hz, 2H, Ph), 7.4 (s, 1H, OH), 4.4 (S, 1H, CH). Anal. Calcd for $C_{16}H_{11}BrN_2O_4$: C, 51.2; H, 2.93; N, 7.467. Found: C, 51.42; H, 2.77; N, 7.49.

7-Bromo-3'-(4-methoxyphenyl)spiro[1,4-benzodiazepine-3,2'-oxirane] -2,5(3H,4H)-dione 5c
Yield 70%, pale yellow crystals; mp 140°C; IR (KBr) γ cm^{-1}: 3560, 3528, 3011, 2980, 1770 and 1651. ^{1}H-NMR (DMSO-d_6) δ 9.96 (s, 1H, NH), 9.8 (s, 1H, NH), 7.6 (s, 1H, Ph), 7.53 (d, J = 5.2 Hz, 2H, Ph), 7.26 (d, J = 6.3 Hz, 2H, Ph), 7.22 (d, J = 4 Hz, 2H, Ph), 4.5 (S, 1H, CH), 3.3 (S, 3H, CH_3). Anal. Calcd for $C_{17}H_{13}BrN_2O_4$: C, 52.44; H, 3.34; N, 7.19. Found: C, 51.92; H, 3.57; N, 7.19.

7-Bromo-3'-(3,4-dihydroxyphenyl)spiro[1,4-benzodiazepine-3,2'-oxirane]-2,5(3H,4H)-dione 5d
Yield 80%, pale yellow crystals; mp 110-112°C; MS m/z 390, 392 (M^+, M^+ + 2); IR (KBr) γ cm^{-1}: 3560, 3528, 3508, 3078, 2990, 1772 and 1681. ^{1}H-NMR (DMSO-d_6) δ 9.9 (s, 1H, NH), 9.8 (s, 1H, NH), 7.6 (s, 1H, Ph), 7.53 (d, J = 4.34 Hz, 2H, Ph), 7.24 (s, 1H, Ph), 7.19 (d, J = 6.2 Hz, 2H, Ph), 7.4 (s, 1H, OH), 7.3 (s, 1H, OH), 4.4 (S, 1H, CH). Anal. Calcd for $C_{16}H_{11}BrN_2O_5$: C, 49.10; H, 2.81; N, 7.16. Found: C, 48.90; H, 2.60; N, 7.18.

7-Bromo-3'-(4-hydroxy-3-methoxyphenyl)spiro[1,4-benzodiazepine-3,2'-oxirane]-2,5(3H,4H)-dione 5e
Yield 75%, pale yellow crystals; mp 106-108°C; IR (KBr) γ cm^{-1}: 3560, 3550, 3074, 2983, 1766 and 1683. ^{1}H-NMR (DMSO-d_6) δ 9.96 (s, 1H, NH), 9.92 (s, 1H, NH), 7.5 (s, 1H, Ph), 7.4 (s, 1H, Ph), 7.3 (d, J = 7.1 Hz, 2H, Ph), 7.16 (d, J = 3 Hz, 2H, Ph), 7.4 (s, 1H, OH), 2.5 (s, 3H, CH_3), 3.8 (S, 1H, CH). Anal. Calcd for $C_{17}H_{13}BrN_2O_5$: C, 50.37; H, 3.21; N, 6.91. Found: C, 50.42; H, 3.27; N, 6.99.

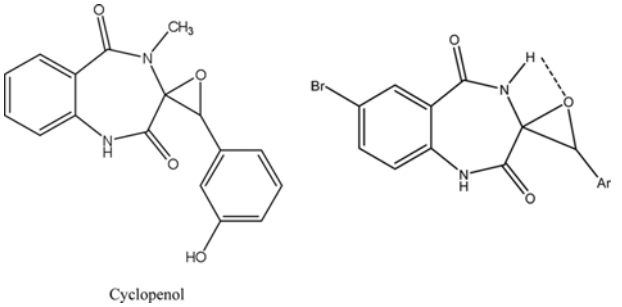
7-Bromo-3'-(3-hydroxyphenyl)spiro[1,4-benzodiazepine-3,2'-oxirane] -2,5(3H,4H)-dione 5f
Yield 40%, pale yellow crystals; mp 120-122°C; MS m/z 374, 376 (M^+, M^+ + 2); IR (KBr) γ cm^{-1}: 3560, 3528, 3064, 2877, 1760 and 1681. ^{1}H-NMR (DMSO-d_6) δ 9.92 (s, 1H, NH), 9.8 (s, 1H, NH), 7.6 (d, J = 3.2 Hz, 2H, Ph), 7.53 (d, J = 4.1 Hz, 2H, Ph), 7.24 (d, J = 5.2 Hz, 2H, Ph), 7.17 (d, J = 6 Hz, 2H, Ph), 7.4 (s, 1H, OH), 4.0 (S, 1H, CH). Anal. Calcd for $C_{16}H_{11}BrN_2O_4$: C, 51.2; H, 2.93; N, 7.467. Found: C, 51.52; H, 2.77; N, 7.19.

7-Bromo-3'-(furan-2-yl)spiro[1,4-benzodiazepine-3,2'-oxirane]-2,5(3H,4H)-dione 5g
Yield 60%, grey crystals, mp 220-222°C; MS m/z 348, 350 (M^+, M^+ + 2); IR (KBr) γ cm^{-1}: 3427, 3419, 3264, 3253, 2980, 1766 and 1683. ^{1}H-NMR (DMSO-d_6) δ 9.92 (s, 1H, NH), 9.9 (s, 1H, NH), 8.14 (d, J = 4 Hz, 2H, furyl), 8.0 (s, 1H, Ph), 7.7 (s, 1H, Ph), 7.6 (s, 1H, Ph), 7.4 (d, J = 2.2 Hz, 1H, furyl), 4.1 (S, 1H, CH). Anal. Calcd for $C_{14}H_9BrN_2O_4$: C, 48.14; H, 2.85; N, 8.02. Found: C, 48.00; H, 2.44; N, 8.12.

Pharmacological activity

In vitro testing

The main objective of this investigation is to compare the acetylcholine potentiating action of different tested compounds with acetylcholinesterase inhibitor physostigmine, on the frog rectus abdominis muscle and guinea pig ileum (Patil et al., 2004).



Cyclopenol

Fig. 2. Design of the new compounds showing H-bond between oxygen molecule of oxirane and N4.

Approximately, 2 cm of guinea pig terminal ileum and frog rectus abdominis muscle were removed and were then placed in organ-bath containing Ringer's solution at room temperature, in case of frog rectus abdominis muscle, or containing tyrod's solution at 37°C, in case of guinea pig terminal ileum. The contractions of the tissues were recorded on a kymograph. Each tissue was left to equilibrate before adding any drug to the organ-bath. The bath solution was aerated with oxygen (95%):carbon dioxide (5%).

Dose-response curves were obtained by increasing the concentration of acetylcholine (100 mg/L)/(10 mg/L) in case of frog rectus abdominis muscle and guinea pig terminal ileum respectively, until a maximal response was reached, then, a sub- maximal dose was selected. All tested compounds were prepared in the concentration 0.1, 0.2, 0.4, 0.8, 1.6 mg and were dissolved in water (1 mL), then added to the organ bath and incubated for 5 min before addition of the selected sub maximal dose of acetylcholine.

On both tissues, physostigmine and new compounds potentiated acetylcholine responses in a dose response manner recorded the best effect in compounds **5b**, **5c** and **5g** that doubled the response of acetylcholine. On the other hand, compounds **5a**, **5e**, **5d** and **5f** had lesser effect on acetylcholine response on both tissues.

Results were recorded according to difference in contractility occurred in both muscles, due to the tested compound from the sub-maximal dose of acetylcholine and were calculated as percentage related to the original increase occurred due to the effect of acetylcholine alone.

In vivo testing

Male albino Wistar rats (200-250 g) were used in the present experiment. Animals were kept under standard laboratory conditions, maintained on a 12/12-h light/dark cycle. Food and water were available *ad libitum* until the beginning of the experiment. Animals were randomly assigned to 9 treatment groups (n = 6) divided as follows; Group I: Rats received vehicle (1% tween 80, orally) and served as normal control group. Group II: Rats received Tacrine (10 mg/kg, orally) and served as standard control group. Groups III- IX: Rats received compounds **5a**-**g** (10 mg/kg, orally). One hour after drug administration, animals were euthanized under deep ether anesthesia by decapitation. Brains were removed and homogenized immediately in ice-cold saline to obtain 10% (w/v) homogenate using glass homogenizer (glas-Col homogenizer). The homogenate was centrifuged at 15,000 rpm for 20 min and the supernatant was used for estimation of acetylcholinesterase activity using colorimetric kinetic Kit (Bio-

Table I. Effect of the synthesized compounds (**5a**-**g**) on frog rectus abdominis and guinea pig ileum compared with physostigmine

Compound	Dose in mg	Frog rectus abdomenis		Guinea pig ileum	
		Increase in contractility in cm	% Increase in contractility	Increase in contractility in cm	% Increase in contractility
5a	0.4	0.2 ± 0.02	13.3	0.4 ± 0.02	10.3
	0.8	0.4 ± 0.03	28.6	0.6 ± 0.05	15.5
	1.2	0.4 ± 0.03	30.8	0.7 ± 0.05	33
	1.6	0.7 ± 0.04	53.3	0.8 ± 0.06	60
5b	0.4	1.0 ± 0.09	62.5	0.6 ± 0.01	41.2
	0.8	1.2 ± 0.10	70	1.4 ± 0.01	72
	1.2	1.6 ± 0.12	89	1.8 ± 0.07	90
	1.6	1.8 ± 0.14	90	2.2 ± 0.09	92
5c	0.4	0.9 ± 0.09	22.5	0.6 ± 0.01	23
	0.8	1.1 ± 0.09	27.5	1.4 ± 0.04	52
	1.2	1.8 ± 0.12	48.6	1.4 ± 0.05	56
	1.6	2.3 ± 0.14	60.5	1.6 ± 0.08	62
5d	0.4	0.2 ± 0.01	16.7	0.5 ± 0.01	12
	0.8	0.3 ± 0.03	20	0.7 ± 0.03	18
	1.2	0.3 ± 0.02	30	1.0 ± 0.06	30
	1.6	0.5 ± 0.04	41	1.2 ± 0.08	40
5e	0.4	0.1 ± 0.01	5.3	0.4 ± 0.02	6.2
	0.8	0.2 ± 0.01	10.5	0.5 ± 0.03	11.33
	1.2	0.3 ± 0.02	20	0.8 ± 0.05	22
	1.6	0.4 ± 0.03	28.6	1.2 ± 0.08	35
	2.0	0.5 ± 0.03	36		
5f	0.4	0.2 ± 0.02	9.5	0.6 ± 0.03	9.9
	0.8	0.2 ± 0.01	10	0.7 ± 0.05	11
	1.2	0.2 ± 0.01	11.11	0.8 ± 0.07	20
	1.6	0.4 ± 0.03	25	1.0 ± 0.07	29
5g	0.4	0.8 ± 0.06	19.5	1.0 ± 0.09	32.3
	0.8	1.3 ± 0.09	36	2.6 ± 0.12	72.2
	1.2	2.0 ± 0.14	59	2.6 ± 0.11	80
	1.6	2.3 ± 0.17	67.6	2.8 ± 0.13	85
Physostigmine	0.4	0.1 ± 0.01	6	0.6 ± 0.02	37
	0.8	0.8 ± 0.04	30	1.0 ± 0.06	60
	1.2	1.1 ± 0.09	70	1.2 ± 0.09	70
	1.6	1.6 ± 0.10	80	1.4 ± 0.12	80

diagnostic) (Mora et al., 1999).

The principle of the Assay is based on that the thioester substrate acetylthiocholine (AchSC) is hydrolyzed by the enzyme, releasing a sulfhydrylic group able to react with Bis (3-carboxy-4-nitrophenyl) disulfide (Ellman's reagent) (Ellman, 1959). The kinetics of this activity is then followed with the use of a spectrophotometer at 412 nm for 2 min. Absorbance is measured at 0, 1 and 2 min and the mean change in absorbance (δA) is calculated for each sample the values were recorded (Table III).

Acute toxicity and lethality (LD_{50}) testing

Swiss albino mice were used; the mice were divided into groups of eight. Each group was injected intra peritoneal with a dose of the compounds **5a**-**g**, starting by 20 mg/kg (active dose of diazepam); no mortality was observed among mice. The dose was multiplied and further 40, 60, 80, 100 and 200 mg/kg doses were injected, but still no mortality among the tested mice was observed. This proved the high safety profile of the new compounds.

RESULTS

The derivatives were tested for their activity as cholinesterase inhibitors and were found to be active as reversible cholinesterase inhibitors. Compounds showed safety profile through LD_{50} testing which is reached up to 200 mg/kg. Compound **5b** was found to

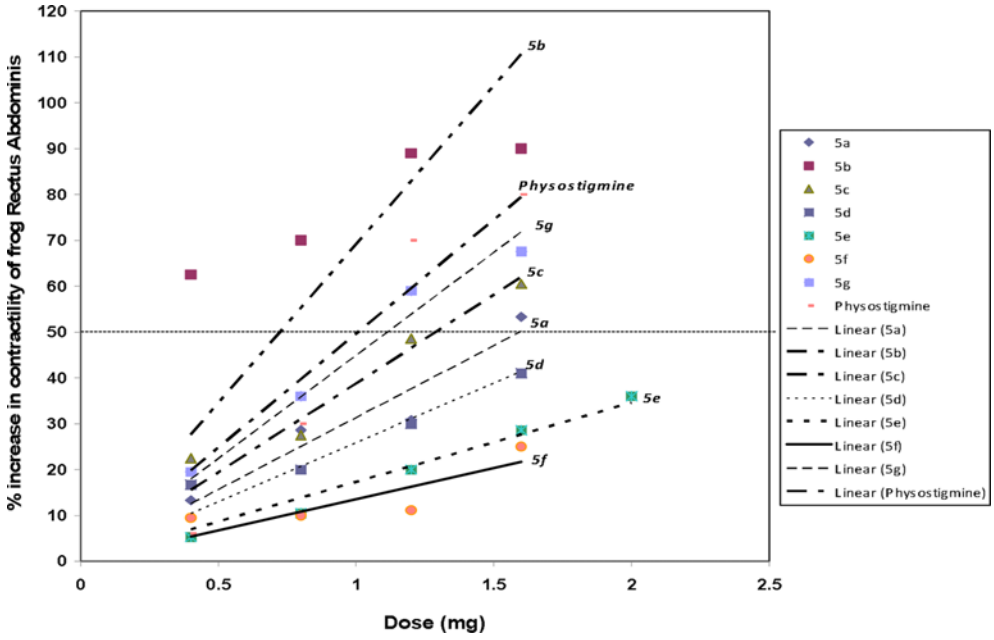


Fig. 3. Graph representing dose response curve of the tested compounds (**5a**-**g**) and physostigmine on frog rectus abdominis.

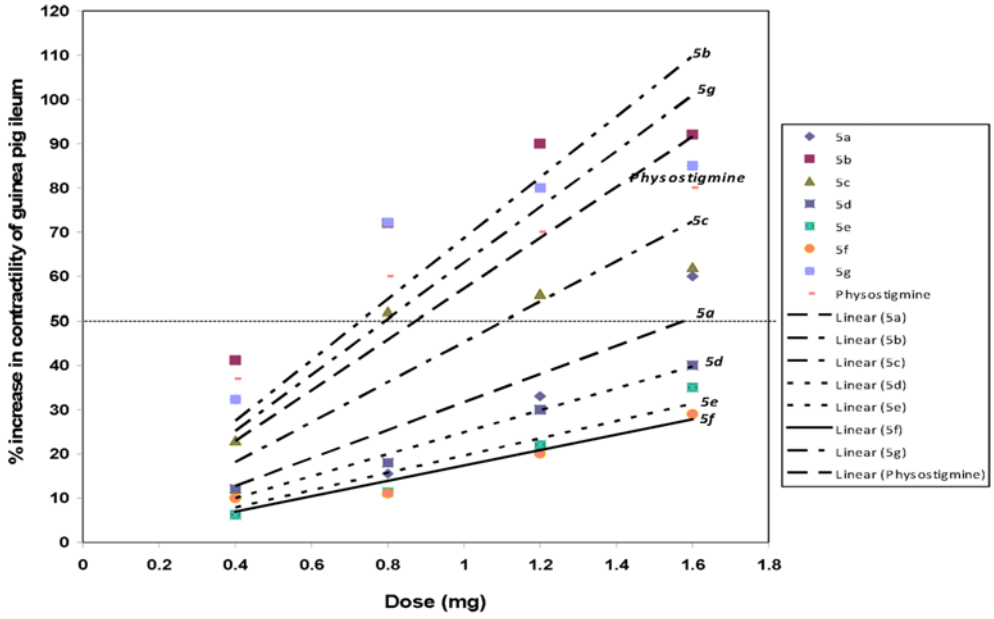


Fig. 4. Dose response curve of the tested compounds (**5a**-**g**) and physostigmine on guinea pig ileum.

be more active than the reference compound, physostigmine it increased muscle contraction up to 90% and 92% while physostigmine showed only 80% increase in muscle contraction. It also showed similar cholinesterase level as tacrine when tested using colorimetric kinetic kit both showed 63% inhibition. Compounds **5a**, **5c** and **5g** exhibited moderate activity towards both muscles where they showed 53, 60 and 67% increase in muscle contraction of frog rectus abdominis while physostigmine increased contraction by 80%. Moreover they increased contractility of guinea pig ileum by 60, 62 and 85% compared to only 80% increase by physostigmine. Inhibition to the enzyme assay by Ellman's procedure showed 60, 56 and 60% inhibition of compounds **5a**, **5c** and **5g** compared to 63% inhibition by tacrine. Compounds **5d**, **5e** and **5f** were found to have a lesser value of cholinesterase inhibitor activity showing 41, 36 and 25% increase in frog rectus abdominis contraction and 40, 35 and 29% increase in contractility of guinea pig muscle. On the

Table II. ED_{50} measured for tested compounds and physostigmine

Frog Rectus Abdominis		Guinea Pig Ileum	
Compound	ED_{50}	Compound	ED_{50}
5b	0.723	**5b**	0.729
Physostigmine	1.007	**5g**	0.793
5g	1.113	Physostigmine	0.873
5c	1.289	**5c**	1.105
5a	1.595	**5a**	1.578
5d	1.931	**5d**	2.013
5e	2.889	**5e**	2.555
5f	3.685	**5f**	2.886

Table III. Effect of Tacrine and new 1,4-benzodiazepine derivatives on brain acetyl choline esterase content in rats

Groups	Choline Esterase content (U/gm wet weight)	% Inhibition
Normal control (Saline)	253.03 ± 24.26	0
Tacrine	93.19 ± 11.17*	63.17
5a	101.66 ± 6.54*	60.08
5b	91.89 ± 8.93*	63.68
5c	110.79 ± 4.21*	56.21
5d	152.48 ± 4.28*,#	39.74
5e	125.12 ± 11.19*	50.55
5f	158.36 ± 18.11*,#	37.41
5g	99.98 ± 8.73*	60.48

Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparisons test for comparison of means of different groups. Each value represents mean ± S.E. (n = 6 rats). *Significantly different from normal control group at $p < 0.05$. #Significantly different from tacrine group at $p < 0.05$.
% Inhibition = 100 [(mean of group/mean of control) × 100].

other hand, **5d**, **5e** and **5f** showed 39.74, 50.55 and 37.41% inhibition by enzyme assay but still retain activity. From the previous results, parasubstitution increased activity more than disubstitution and to a great effect more than m- substitution which resembles the cyclopenol. The hydroxyl group is far more active than methoxy or chloro derivatives. The removal of the methyl group from N_4 enhanced activity and on the other hand increased the rigidity of the molecule.

DISCUSSION

Chemistry

The target of this paper was to prepare 1,4-benzodiazepine derivatives analogous to Cyclopenin using 4*H*-3,1-benzoxazin-2,4-dione (Isatoic anhydride) **1** and Glycine as starting materials for the cyclization to compound **2** (Mohiuddin et al., 1985). Compound **2** was suspended in acetic acid and bromine solution was added to give the 7-bromo derivative **3** (Osman et al., 2002). Condensation of **3** with a number of aromatic aldehydes in acetic anhydride afforded the 3-benzylidine 7-bromo-3,4-dihydro-1*H*-benzo[e][1,4] diazepine-2,5-dione derivatives **4** these compounds showed distinctive singlet peaks at values from δ 6.5-7.6 ppm in NMR charts equivalent to the benzylidene proton in addition to the distinct peaks equivalent to both NH hydrogens at 9.8-10.04 ppm which were found to be D_2O exchangeable. The separation of E and Z diastereomers can be performed by the use of solvent (Markoviæ et al., 2000). In this case TLC showed two spots, one major, which was separated by the use of ethyl acetate as the solvent to give pure **4** derivatives. Oxidation of **4** by the use of peroxybenzoic acid to give the epoxy derivative, 7-Bromo-3'-phenyl spiro [1,4-benzodiazepine-3,2'-oxirane]-2,5(3H,4H)-dione derivatives **5** in good yield. These derivatives showed a single peak at δ 3.8-4.5 ppm in NMR equivalent to the oxirane H which could be explained according to the possible formation of hydrogen bond between Oxygen atom in oxirane and hydrogen of the unsubstituted Nitrogen at position 4 leading to only one possible conformation of the produced derivatives (Fig. 2).

The structures of the target compounds were characterized by ^{1}H-NMR, IR, mass, microanalysis and the completion of the reaction was confirmed by TLC.

Pharmacology

During the synthesis of the new compounds several changes were performed on the original structure of cyclopenin and cyclopenol in order to increase their AChE inhibitory activity. First, the nitrogen atom at position 4 was prepared without the methyl group; on the other hand NH group participated in the purity of the final compounds (Fig. 2) which furthermore would lead to increase in its pharmacological activity. Second, a bromine atom was introduced at position 7, the effect of introducing halogen atom at position 7 was to be considered, as the activity of 1,4-benzodiazepine derivatives increases by halogens and other electron-withdrawing substituents at position 7 (Scholl et al., 1983). Besides, bromination at position 6, 8 or 9 leads to complete loss of pharmacological activity (Sternbach, 1971). Furthermore, the involvement of the basal forebrain cortical cholinergic system was seen to play a crucial role in the cognitive aspects of anxiety (Bernston et al., 1998).

The seven new compounds **5a**-**g** were subjected to preliminary pharmacological screening and were tested for their cholinesterase inhibitor activity against physostigmine in *vitro* and tacrine in *vivo* as reference.

Acetylcholine was freshly prepared, and the test was performed in *vitro*. The acetylcholine potentiation induced contraction was measured after each addition on frog rectus abdominis muscle and guinea pig ileum and the compounds were found to elevate acetylcholine effect. The effect of the compounds diminished after washing frog rectus abdominis with fresh Ringer's solution and washing guinea pig ileum with fresh Tyrode solution suggesting that their effect was reversible. The dose was determined according to the effective dose of diazepam as cholinesterase inhibitor (20 mg/kg) (Tonkopii et al., 1978). Moreover, male rates were injected with the compounds and with tacrine, the level of cholinesterase enzyme was measured and percentage inhibition of the enzyme was calculated. All the compounds degraded the level of cholinesterase enzyme with values close to the standard tacrine. In another approach male albino mice were divided into groups of eight. The effective dose (20 mg/kg) was injected *in vivo* in mice to measure the LD_{50} of the compounds. It caused no mortality, so it was multiplied several times till up to 200 mg/kg, none of the tested mice died, which proved their high safety.

ACKNOWLEDGEMENTS

We would like to thank Prof. Dr. Khaled Abuzid and Dr. Nahla Farag for their help and support.

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