Asian Journal of Pharmaceutical and Health Sciences
www.ajphs.com

Synthesis, Characterization and Enzyme Inhibition Studies on Various *O*-Substituted Derivatives of *N*-(4-Hydroxyphenyl)-*N*-methyl-*O*-phenyl carbamate

Muhammad Athar Abbasi[1]*, Abdul Hafeez[1], Aziz-ur-Rehman[1], Khalid Mohammed Khan[2], Muhammad Ashraf[3], Syeda Abida Ejaz[4]

1 Department of Chemistry, Government College University, Lahore-54000, Pakistan.
2 HEJ Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan
3 Department of Biochemistry and Biotechnology;
4 Department of Pharmacy; The Islamia University of Bahawalpur, Bahawalpur-63100, Pakistan.

ARTICLE HISTORY

Received: 27.04.2012

Accepted: 18.07.2012

Available online: 10.11.2012

Keywords:

4-(Aminomethyl)phenol, Phenylchloroformate, carbamate, butyryl cholinesterase, and ^{1}H-NMR

*Corresponding author:

Email : atrabbasi@yahoo.com
Tel : (+92)-42-111000010 Ext.266.

ABSTRACT

In the present study, a series of *O*-substituted carbamates have been synthesized. The reaction of 4-(aminomethyl) phenol (1) with phenylchloroformate (2) yielded *N*-(4-hydroxyphenyl)-*N*-methyl-*O*-phenyl carbamate (3). This product 3 on treatment with alkyl/aryl halides in the presence of lithium hydride yielded ten different *O*-substituted carbamates. All newly synthesized compounds were characterized by IR, EI-MS and ^{1}H-NMR spectra and then screened against α-chymotrypsin, acetylcholinesterase, and butyrylcholinesterase enzymes. The results revealed that *N*-(4-benzoxyphenyl)-*N*-methyl-*O*-phenyl carbamate (8b), *N*-[4-(2-chlorobenzoxy)phenyl]-*N*-methyl-*O*-phenyl carbamate (8c), and *N*-[4-(4-chlorobenzoxy)phenyl]-*N*-methyl-*O*-phenyl carbamate (8e) exhibited good inhibitory potential against acetylcholinesterase and butyrylcholinesterase and are possible target molecule for the treatment of Alzheimer's disease.

INTRODUCTION

Carbamates are not only pharmacologically but also synthetically important class of organic compounds. Carbamates are used in the synthesis of polymers (e.g., polyurethane), herbicides, fungicides, pesticides, and pharmaceuticals. The carbamate functionality containing molecules have been reported to posses antibacterial, antiepileptic, anticonvulsants, anticancer and enzyme inhibitory properties [1,2]. They also function as intermediates in the synthesis of fine chemicals [3]. These are excellent templates for the synthesis of CC, and carbonheteroatom bonds as well as powerful protecting groups [4,5]. They also function as chiral auxiliaries [6]. Carbamates have also been reported to be inhibitors of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) enzymes which degrade two major endocannabinoid transmitters anandamide and 2-arachidonoylglycerol, respectively, without causing psycotropic behavioral effects. Carbamates' inhibition of FAAH and MAGL displayed analgestic and anxiolytic properties in rodents [7,8]. Carbamates can be detected through measurement of bio-electrical signals caused by the inhibition of acetylcholinesterase (AChE) because carbamates are strong inhibitors of AChE. Within the nervous system of insects and mammals acetylcholinesterase terminates impulse transmission at cholinergic synapses through rapid hydrolysis of acetylcholine which is neurotransmitter. So, accumulation of acetylcholine blocks signal processing property of nerves [9]. The (−)S-*N*-ethyl-3-[(1-dimethyl-amino)ethyl]-*N*-methylphenylcarbamate hydrogen tartrate, commonly known as Rivastigmine hydrogen tartrate, is a carbamate inhibitor of acetylcholinesterase (AChE) that is used in the treatment Alzheimer's disease [10-12]. Disubstituted thiadiazole carbamates have been reported to treat Niemann-Pick type C (NPC) disease to control accumulation of cholesterol at cellular level [13]. Carbamates are also active against amoebiasis [14], brain as well as breast cancer [15,16] and are used as prodrug to increase the bioavailability of drugs in the body of organisms [17]. Acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) consist of an enzyme family which includes serine hydrolases. The diverse specificities for the substrates and inhibitors for these enzymes are due to the differences in amino acid residues of the active sites of AChE and BChE. Actually the system of enzyme is responsible for the termination of acetylcholine at cholinergic synapses. These are key components of cholinergic brain synapses and neuromuscular junctions. The major function of AChE and BChE is to catalyze the hydrolysis of the neurotransmitter acetylcholine and termination of the nerve impulse in cholinergic synapses [18,19]. It has been found that BChE is present in appreciably

higher quantity in Alzheimer's plaques than in the normal age linked dementia of brains. H_1 and H_2 receptor antagonists possess AChE inhibitory activities. Cholinesterase inhibitors raise the quantity of acetylcholine available for neuronal and neuromuscular transmission through their ability to reversibly or irreversibly. Hence, the search for new cholinesterase inhibitors is considered an important and ongoing strategy to introduce new drug candidates for the treatment of Alzheimer's disease and other related diseases [20,21].

Literature survey revealed that minor structural modifications in carbamate moiety containing molecules can lead to quantitative as well as qualitative changes in the biological activities. These findings impelled us to synthesize the various *O*-substituted derivatives of *N*-(4-hydroxyphenyl)-*N*-methyl-*O*-phenyl carbamate and subsequent their biological screening with the aim of searching valuable cholinestrases inhibitors.

Experimental Protocol Chemistry

Synthesis of *N*-(4-hydroxyphenyl)-N-methyl-*O*-phenyl carbamate (3)

4-(Aminomethyl)phenol (metol; 1; 2.755 g, 8 mmol) was suspended in cold water. Little methanol was added to effect solubility of metol. The pH of suspension was adjusted to 8 with concentrated aqueous solution of $NaHCO_3$. Phenylchloroformate (2; 2 mL, 16 mmol) was added drop by drop with constant stirring of the reaction mixture, placed in ice cold water. The completion of the reaction was monitored with TLC. The resulting grayish precipitate were filtered, washed with distilled water, and dried to get the titled compound 3. Yield 79%.

Synthesis of *O*-phenyl-*O*-(4-*N*-methylphenoxyformamido phenyl) carbonate (6a)

In the concentrated solution of NaOH, 3 (0.2 g, 0.8 mmol) was dissolved with vigorous shaking. While maintaining pH at 12, shaking was continued after addition of phenylchloroformate (2; 0.1 mL, 0.8 mmol) for 30-45 min. The completion of reaction was monitored via TLC. Filtered the precipitate formed, washed and dried to afford 6a. Yield 83%.

Synthesis of *N*-(4-benzoyloxyphenyl)-*N*-methyl-*O*-phenyl carbamate (6b)

In the concentrated solution of NaOH, dissolved 3 (0.8 mmol) with vigorous shaking, maintained pH at 12 and continued shaking after addition of benzoyl chloride 4 (0.1 mL, 0.8 mmol) for 30-45 min. After monitoring the completion of reaction via TLC, the sticky semisolid reddish product was separated and washed with distilled water. Yield 78%.

Synthesis of *N*-(4-acetoxyphenyl)-*N*-methyl-*O*-phenyl carbamate (6c)

0.2 g (0.8 mmol) compound 3 was dissolved in concentrated aqueous solution of NaOH, and adjusted the pH at 12. Acetic anhydride (1.0 mL) was added gradually in the reaction mixture for the acetylation of 3. The reaction mixture was vigorously shaken for 20 min and the progress of the reaction was monitored by TLC. The product was sticky oily maroon colored that was separated, washed and dried to afford *N*-(4-acetoxyphenyl)-*N*-methyl-*O*-phenyl carbamate (6c). Yield 75%.

General procedure for the synthesis of *N*-(4-alkyl/aryloxyphenyl)-*N*-methyl-*O*-phenyl carbamates (8a-8g)

The calculated amount of 3 (0.8 mmol) was taken in the round bottom flask (50 mL), then *N*,*N*-dimethylformamide DMF (7 mL) was added to dissolve it followed by the addition of lithium hydride (0.8 mmol) to the mixture. The mixture was stirred for 30 min at room temperature, and then slowly added the calculated amount of alkyl/aryl halide (7a-g, 0.8 mmol) to the solution, and the mixture was further stirred for 2 to 3 hours at room temperature. The end of reaction was observed with TLC till single spot. The product was precipitated by adding water. It was filtered, washed with distilled water and crystallized from suitable solvent.

METHODOLOGY

TLC was performed on pre-coated silica gel G-25-UV_{254} plates. Detection was carried out at 254 nm, and by ceric sulphate reagent. Purity was checked on TLC with different solvent systems using ethyl acetate and *n*-hexane giving single spot. The IR spectra were recorded in KBr on a Jesco-320-A spectrophotometer (wave number in cm^{-1}). ^{1}H-NMR spectra was recorded in CD_3OD and $CDCl_3$ on a Bruker spectrometers operating at 500 MHz and 400 MHz. Chemical shifts are given in ppm. EI-MS were recorded on a JMS-HX-110 spectrometer, with a data system. The melting points were recorded on a Griffin & George melting point apparatus by open capillary tube and were uncorrected.

SPECTRAL CHARACTERIZATION OF THE SYNTHESIZED CARBAMATES

***N*-(4-Hydroxyphenyl)-*N*-methyl-*O*-phenyl carbamate (3)**

Grayish amorphous solid, m.p 125-126°C, Yield 79%. IR (KBr): λ_{max}: 3267 (-OH), 3041 (Ar-H), 1697 (C=O), 1350, 1280 (C-N), 1262 (C-O-C); ^{1}H-NMR (500 MHz, CD_3OD): δ 3.27 (s, 3H, -NCH_3), 6.80 (d, *J* = 8.5 Hz, 2H, H-3, H-5), 7.18 (d, *J* = 9.0 Hz, 2H, H-2, H-6), 7.36-7.33 (m, 5H, H-2'- H-6'); EIMS: *m/z* 243 $[M]^+$, 228 $[M\text{-}CH_3]^+$, 150 $[M\text{-}C_6H_5O]^+$, 122 $[M\text{-}C_6H_5OCO]^+$, 121 $[C_6H_5OCO]^+$, 94 $[C_6H_5OH]^+$, 77 $[C_6H_5]^+$, 51 $[C_4H_3]^+$.

***O*-Phenyl-*O*-(4-*N*-methylphenoxyformamidophenyl) carbonate (6a)**

Tea pink colored shiny amorphous solid, m.p 126-128°C, Yield 83%. IR (KBr): λ_{max}: 3036 (Ar-H), 1692, 1775 (C=O), 1337, 1281 (C-N), 1279, 1057 (C-O-C); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 3.41 (s, 3H, -NCH_3), 7.30- 7.25 (m, 8H, H-3, H-5, H-2', H-4', H-6', H-2", H-4", H-6"), 7.41 (br t, *J* = 8.0 Hz, 4H, H-3', H-5', H-3", H-5"), 7.98 (d, *J* = 8.5 Hz, 2H, H-2, H-6); EIMS: *m/z* 363 $[M]^+$, 348 $[M\text{-}CH_3]^+$, 270 $[M\text{-}C_6H_5O]^+$, 242 $[M\text{-}C_6H_5OCO]^+$, 121 $[C_6H_5OCO]^+$, 94 $[C_6H_5OH]^+$, 77 $[C_6H_5]^+$, 51 $[C_4H_3]^+$.

***N*-(4-Benzoyloxyphenyl)-*N*-methyl-*O*-phenyl carbamate (6b)**

Dark brown sticky shiny solid, m.p 73-80°C, Yield 78%. IR (KBr): λ_{max}: 3042 (Ar-H), 1695, 1730 (C=O), 1339, 1277 (C-N), 1271, 1061 (C-O-C); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 3.43 (s, 3H, -NCH_3), 7.25 (d, *J* = 9.0 Hz, 2H, H-3, H-5), 7.65-7.16 (m, 7H, H-2'-H-6', H-3", H-5"), 8.02 (dd, *J* = 7.0, 1.5 Hz, 1H, H-4"), 8.10 (d, *J* = 9.5 Hz, 2H, H-2, H-6), 8.20 (dd, *J* = 7.0, 1.5 Hz, 2H, H-2", H-6"); EIMS: *m/z* 347 $[M]^+$, 332 $[M\text{-}CH_3]^+$, 270 $[M\text{-}C_6H_5]^+$, 254 $[M\text{-}C_6H_5O]^+$, 242 $[M\text{-}C_6H_5CO]^+$, 226 $[M\text{-}C_6H_5OCO]^+$, 121 $[C_6H_5OCO]^+$, 105 $[C_6H_5CO]^+$, 94 $[C_6H_5OH]^+$, 77 $[C_6H_5]^+$, 51 $[C_4H_3]^+$.

***N*-(4-Acetoxyphenyl)-*N*-methyl-*O*-phenyl carbamate (6c)**

Maroon colored sticky liquid, Yield 65%. IR (KBr): λ_{max}: 3041 (Ar-H), 1690, 1725 (C=O), 1360 (CH_3-CO), 1348, 1272 (C-N), 1263 (C-O-C), 1250 (Ac-O); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 2.29 (s, 3H, CH_3-1''), 3.39 (s, 3H, -NCH_3), 7.10 (d, *J* = 8.5 Hz, 2H, H-3, H-5), 7.17 (br t, *J* = 7.5 Hz, 3H, H-2', H-4', H-6'), 7.33 (merged t, *J* = 7.5 Hz, 2H, H-3', H-5'), 7.35 (merged d, *J* = 7.0 Hz, 2H, H-2, H-6); EIMS: *m/z* 285 $[M]^+$, 270 $[M-CH_3]^+$, 242 $[M-COCH_3]^+$, 192 $[M-C_6H_5O]^+$, 164 $[M-C_6H_5OCO]^+$, 121 $[C_6H_5OCO]^+$, 94 $[C_6H_5OH]^+$, 77 $[C_6H_5]^+$, 51 $[C_4H_3]^+$, 43 $[CH_3CO]^+$.

***N*-(4-Ethoxyphenyl)-*N*-methyl-*O*-phenyl carbamate (8a)**

Light mustard colored amorphous solid, m.p 74-75°C, Yield 76%. IR (KBr): λ_{max}: 3040 (Ar-H), 1697 (C=O), 1348, 1278 (C-N), 1261, 1060 (C-O-C); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 1.39 (t, *J* = 7.0 Hz, 3H, CH_3-2''), 3.33 (s, 3H, -NCH_3), 4.01 (q, *J* = 7.0 Hz, 2H, CH_2-1''), 6.88 (d, *J* = 8.5 Hz, 2H, H-3, H-5), 7.23-7.15 (m, 5H, H-2'- H-6'), 7.31 (d, *J* = 8.5 Hz, 2H, H-2, H-6); EIMS: *m/z* 271 $[M]^+$, 256 $[M-CH_3]^+$, 242 $[M-C_2H_5]^+$, 178 $[M-C_6H_5O]^+$, 150 $[M-C_6H_5OCO]^+$, 121 $[C_6H_5OCO]^+$, 94 $[C_6H_5OH]^+$, 77 $[C_6H_5]^+$, 51 $[C_4H_3]^+$.

***N*-(4-Benzoxyphenyl)-*N*-methyl-*O*-phenyl carbamate (8b)**

Camel colored amorphous solid, m.p 86.6-87°C, Yield 80%. IR (KBr): λ_{max}: 3038 (Ar-H), 1695 (C=O), 1345, 1282 (C-N), 1250, 1062 (C-O-C); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.34 (s, 3H, -NCH_3), 5.05 (s, 2H, CH_2-7''), 6.96 (d, *J* = 8.8 Hz, 2H, H-3, H-5), 7.15 (t, *J* = 8.4 Hz, 1H, H-4'), 7.23 (br d, *J* = 8.8 Hz, 2H, H-2', H-6'), 7.42-7.29 (m, 7H, H-3', H-5', H-2''- H-6''), 7.92 (d, *J* = 8.8 Hz, 2H, H-2, H-6); EIMS: *m/z* 333 $[M]^+$, 318 $[M-CH_3]^+$, 256 $[M-C_6H_5]^+$, 242 $[M-CH_2C_6H_5]^+$, 240 $[M-C_6H_5O]^+$, 212 $[M-C_6H_5OCO]^+$, 121 $[C_6H_5OCO]^+$, 94 $[C_6H_5OH]^+$, 77 $[C_6H_5]^+$, 51 $[C_4H_3]^+$.

***N*-[4-(2-Chlorobenzoxy)phenyl]-*N*-methyl-*O*-phenyl carbamate (8c)**

Dark peach colored sticky liquid, Yield 57%. IR (KBr): λ_{max}: 3037 (Ar-H), 1691 (C=O), 1342, 1280 (C-N), 1260, 1065 (C-O-C), 751 (C-Cl); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.36 (s, 3H, -NCH_3), 5.15 (s, 2H, CH_2-7''), 6.98 (d, *J* = 8.8 Hz, 2H, H-3, H-5), 7.15 (t, *J* = 7.2 Hz, 2H, H-3', H-5'), 7.31-7.25 (m, 6H, H-2', H-4', H-6', H-4'', H-5'', H-6''), 7.40 (d, *J* = 8.8 Hz, 1H, H-3''), 7.54 (d, *J* = 8.8 Hz, 2H, H-2, H-6); EIMS: *m/z* 367 $[M]^+$, 352 $[M-CH_3]^+$, 274 $[M-C_6H_5O]^+$, 256 $[M-C_6H_4Cl]^+$, 246 $[M-C_6H_5OCO]^+$, 242 $[M-CH_2C_6H_4Cl]^+$, 125 $[C_6H_4CH_2Cl]^+$, 121 $[C_6H_5OCO]^+$, 94 $[C_6H_5OH]^+$, 77 $[C_6H_5]^+$, 51 $[C_4H_3]^+$.

***N*-[4-(3-Chlorobenzoxy)phenyl]-*N*-methyl-*O*-phenyl carbamate (8d)**

Light tea pink colored amorphous solid, m.p 62-63°C, Yield 73%. IR (KBr): λ_{max}: 3047 (Ar-H), 1692 (C=O), 1340, 1283 (C-N), 1261, 1056 (C-O-C), 750 (C-Cl); ^{1}H-NMR (400 MHz, $CDCl_3$):δ 3.35 (s, 3H, -NCH_3), 5.02 (s, 2H, CH_2-7''), 7.15 (t, *J* = 7.2 Hz, 2H, H-3', H-5'), 7.28-7.26 (m, 6H, H-2', H-4', H-6', H-4'', H-5'', H-6''), 7.29 (s, 1H, H-2''), 7.42 (d, *J* = 8.8 Hz, 2H, H-2, H-6), 7.93 (d, *J* = 8.8 Hz, 2H, H-3, H-5); EIMS: *m/z* 367 $[M]^+$, 352 $[M-CH_3]^+$, 274 $[M-C_6H_5O]^+$, 256 $[M-C_6H_4Cl]^+$, 246 $[M-C_6H_5OCO]^+$, 242 $[M-CH_2C_6H_4Cl]^+$, 125 $[C_6H_4CH_2Cl]^+$, 121 $[C_6H_5OCO]^+$, 94 $[C_6H_5OH]^+$, 77 $[C_6H_5]^+$, 51 $[C_4H_3]^+$.

***N*-[4-(4-Chlorobenzoxy)phenyl]-*N*-methyl-*O*-phenyl carbamate (8e)**

Zinc colored powder, m.p 86-87°C, Yield 81%. IR (KBr): λ_{max}: 3045 (Ar-H), 1689 (C=O), 1343, 1279 (C-N), 1267, 1064 (C-O-C), 747 (C-Cl); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.43 (s, 3H, -NCH_3), 5.01 (s, 2H, CH_2-7''), 6.93 (d, *J* = 8.8 Hz, 2H, H-3, H-5), 7.25-7.13 (m, 5H, H-2', H-4', H-6', H-2'', H-6''), 7.30 (merged d, *J* = 8.0 Hz, 4H, H-3', H-5', H-3'', H-5''), 7.34 (d, *J* = 8.8 Hz, 2H, H-2, H-6); EIMS: *m/z* 367 $[M]^+$, 352 $[M-CH_3]^+$, 274 $[M-C_6H_5O]^+$, 256 $[M-C_6H_4Cl]^+$, 246 $[M-C_6H_5OCO]^+$, 242 $[M-CH_2C_6H_4Cl]^+$, 125 $[C_6H_4CH_2Cl]^+$, 121 $[C_6H_5OCO]^+$, 94 $[C_6H_5OH]^+$, 77 $[C_6H_5]^+$, 51 $[C_4H_3]^+$.

***N*-[4-(4-Bromobenzoxy)phenyl]-*N*-methyl-*O*-phenyl carbamate (8f)**

Camel colored amorphous solid, m.p. 76.8-77.5°C, Yield 86%. IR (KBr): λ_{max}: 3043 (Ar-H), 1688 (C=O), 1346, 1278 (C-N), 1259, 1059 (C-O-C), 601 (C-Br); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.43 (s, 3H, -NCH_3), 4.99 (s, 2H, CH_2-7''), 6.94 (d, *J* = 8.8 Hz, 2H, H-3, H-5), 7.17 (t, *J* = 7.2 Hz, 2H, H-3', H-5'), 7.30-7.23 (m, 5H, H-2', H-4', H-6', H-2'', H-6''), 7.68 (d, *J* = 9.2 Hz, 2H, H-3'', H-5''), 7.70 (d, *J* = 8.8 Hz, 2H, H-2, H-6); EIMS: *m/z* 411 $[M]^+$, 396 $[M-CH_3]^+$, 318 $[M-C_6H_5O]^+$, 290 $[M-C_6H_5OCO]^+$, 256 $[M-C_6H_4Br]^+$, 242 $[M-CH_2C_6H_4Br]^+$, 169 $[C_6H_4CH_2Br]^+$, 121 $[C_6H_5OCO]^+$, 94 $[C_6H_5OH]^+$, 77 $[C_6H_5]^+$, 51 $[C_4H_3]^+$.

***N*-[4-(4-Fluorobenzoxy)phenyl]-*N*-methyl-*O*-phenyl carbamate (8g)**

Camel colored amorphous solid, m.p 68-69°C, Yield 78%. IR (KBr): λ_{max}: 3037 (Ar-H), 1694 (C=O), 1347, 1277 (C-N), 1269 (C-O-C), 1102 (C-F); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.34 (s, 3H, -NCH_3), 5.00 (s, 2H, CH_2-7''), 6.95 (d, *J* = 8.4, 2H, H-3, H-5), 7.36-7.03 (m, 9H, H-2' - H-6', H-2'', H-3'', H-5'', H-6''), 7.40 (d, *J* = 8.4, 2H, H-2, H-6); EIMS: *m/z* 351 $[M]^+$, 336 $[M-CH_3]^+$, 258 $[M-C_6H_5O]^+$, 256 $[M-C_6H_4F]^+$, 242 $[M-CH_2C_6H_4F]^+$, 230 $[M-C_6H_5OCO]^+$, 121 $[C_6H_5OCO]^+$, 94 $[C_6H_5OH]^+$, 77 $[C_6H_5]^+$, 51 $[C_4H_3]^+$.

ENZYME INHIBITION ESSAYS

Acetyl cholinesterase Assay

The AChE inhibition activity was performed according to the method [22] with slight modifications. Total volume of the reaction mixture was 100 µL. It contained 60 µL Na_2HPO_4 buffer with concentration of 50 mM and pH 7.7. 10 µL test compound (0.5 mM $well^{-1}$) was added, followed by the addition of 10 µL (0.005 unit $well^{-1}$) enzyme. The contents were mixed and pre-read at 405 nm. Then contents were pre-incubated for 10 min at 37 ºC. The reaction was initiated by the addition of 10 µL of 0.5 mM $well^{-1}$ substrate (acetylthiocholine iodide), followed by the addition of 10 µL DTNB (0.5 mM $well^{-1}$). After 15 min of incubation at 37 ºC absorbance was measured at 405 nm using 96-well plate reader Synergy HT, Biotek, USA. All experiments were carried out with their respective controls in triplicate. Eserine (0.5 mM $well^{-1}$) was used as a positive control. The percent inhibition was calculated by the help of following equation.

$$\text{Inhibition (\%)} = \frac{Control - Test}{Control} \times 100$$

IC_{50} values were calculated using EZFit Enzyme kinetics software (Perrella Scientific Inc. Amherst, USA).

Butyrylcholinesterase Assay

The BChE inhibition activity was performed according to the method [22,23] with slight modifications. Total volume of the reaction mixture was 100 µL containing 60 µL, Na_2HPO_4 buffer, 50 mM and pH 7.7. 10 µL test compound 0.5 mM $well^{-1}$ was added followed by the addition of 10 µL (0.5 unit $well^{-1}$) BChE (Sigma Inc.). The contents were mixed and pre-read at 405 nm and then pre-incubated for 10 min at 37 ºC. The reaction was initiated by the addition of 10 µL of 0.5 mM $well^{-1}$ substrate (butyrylthiocholine chloride). Followed by the addition of 10 µL DTNB, 0.5 mM $well^{-1}$. After 15 min of incubation at 37 ºC, absorbance was measured at 405 nm using 96-well plate reader Synergy HT, Biotek, USA. All experiments were carried out with their respective controls in triplicate. Eserine (0.5 mM $well^{-1}$) was used as positive control. The percent inhibition was calculated by the help of following equation.

$$\text{Inhibition (\%)} = \frac{Control - Test}{Control} \times 100$$

IC_{50} values were calculated using EZFit Enzyme kinetics software (Perrella Scientific Inc. Amherst, USA).

α-Chymotrypsin Assay

α-Chymotrypsin inhibition assay was carried out according to the reported method [24, 25]. A total volume of 100 µL reaction mixture contained 60 µL of 50 mM Tris-HCl buffer (pH 7.6), 10 µL of 0.5 mM test compound and 15 µL (0.9 units) of enzyme (Sigma, USA) prepared in the above buffer. The contents were mixed, preincubated for 15 min at 37°C and preread at 410 nm. The reaction was initiated by the addition of 15 µL of 1.3 mM substrate, *N*-succinyl phenylalanine-*p*-nitroanilide (Sigma, USA). Absorbance was measured at 410 nm using Synergy HT microplate reader after 30-60 min when absorbance values of uninhibited enzyme assay reached 0.7-0.9. The positive and negative controls were included. All experiments were carried out in triplicate. The percent inhibition was calculated by following equation. Inhibition (%) = (Control Test / Control) x 100. IC_{50} values (concentration at which enzyme inhibition is 50%) were calculated using EZ-Fit Enzyme Kinetics Software (Perrella Scientific Inc. Amherst, USA).

Statistical Analysis

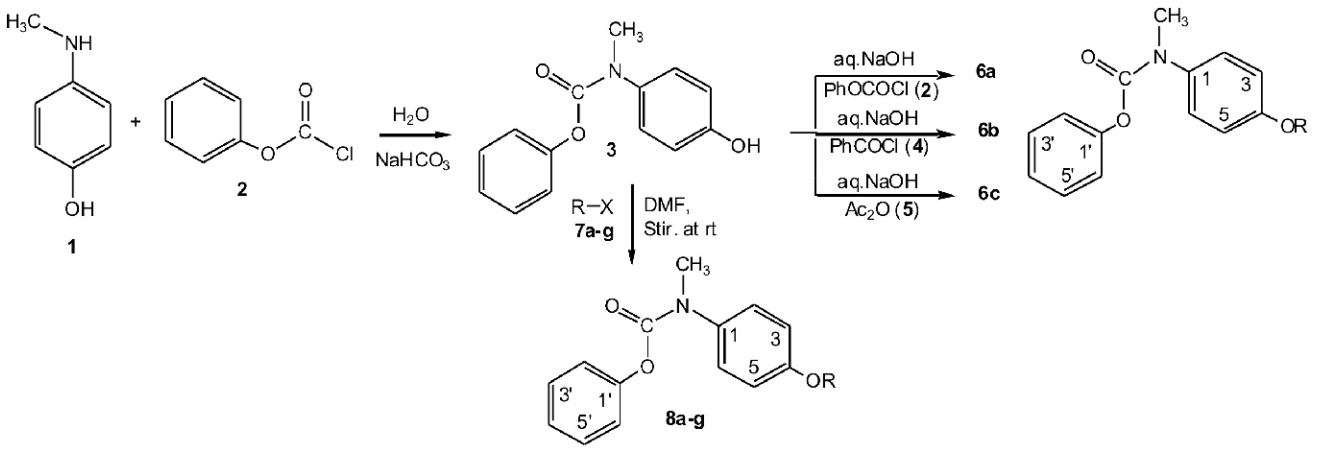
All the measurements were done in triplicate and statistical analysis was performed by Microsoft Excel 2003. Results are presented as mean ± sem.

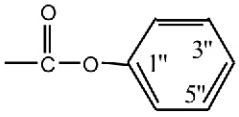
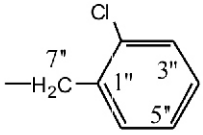
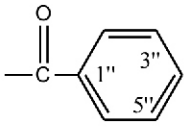
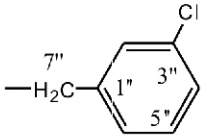
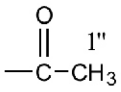
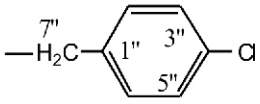
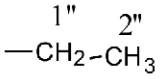
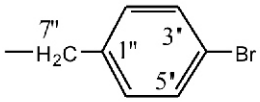
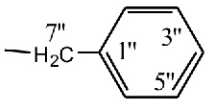
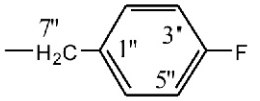
RESULTS AND DISCUSSION

The designed *O*-substituted new derivatives of *N*-(4-hydroxyphenyl)-*N*-methyl-*O*-phenyl carbamate (3) were synthesized according to scheme 1. The parent compound 3 was synthesized as grayish amorphous solid by the reaction of 4-(aminomethyl)phenol (1; metol) and phenylchloroformate (**2**) and its structure was confirmed by its IR, EI-MS and ^{1}H-NMR spectra. The molecular formula $C_{14}H_{13}NO_3$ was established by EI-MS and counting the number of protons in its ^{1}H-NMR spectrum. Its IR spectrum revealed the presence of a hydroxyl group (3267 cm^{-1}) and a carbonyl group (1697 cm^{-1}) in the molecule. The EI-MS showed the molecular ion peak at *m/z* 243 $[M]^+$, which further fragmented into an ion having *m/z* 228 $[M\text{-}CH_3]^+$, showing the presence of a methyl group in the molecule. The base peak was observed at *m/z* 150 $[M\text{-}C_6H_5O]^+$, due to the loss of phenoxy group

Table-1: Evaluation of enzyme inhibitory potential of the carbamates 6a-c and 8a-g. (n = 3, mean±sem)

Sample No.	α-Chymotrypsin		Acetylcholinesterase		Butyrylcholinesterase	
	(%) Inhibition at 0.5mM	IC_{50} (µmol/L)	(%) Inhibition at 0.5mM	IC_{50} (µmol/L)	(%) Inhibition at 0.5mM	IC_{50} (µmol/L)
3	37.64±0.12	NIL	45.65±0.32	NIL	70.52±0.21	141.51±0.15
6a	57.68±0.13	>400	58.41±0.55	>350	61.44±0.54	220.15±0.11
6b	85.77±0.12	149.51±0.04	54.74±0.22	>350	48.92±0.24	NIL
6c	49.81±0.19	NIL	49.52±0.39	>350	68.49±0.33	139.51±0.06
8a	35.39±0.15	NIL	58.22±0.51	>350	70.46±0.15	127.31±0.14
8b	53.93±0.14	>400	71.18±0.66	189.51±0.05	66.96±0.62	180.36±0.09
8c	53.00±0.12	>400	65.38±0.28	224.71±0.14	75.98±0.15	109.32±0.17
8d	46.25±0.11	NIL	64.02±0.56	264.02±0.17	52.86±0.21	>400
8e	41.51±0.13	NIL	80.85±0.20	150.41±0.08	65.95±0.27	191.81±0.21
8f	52.25±0.13	>400	53.97±0.33	>350	45.17±0.31	NIL
8g	65.85±0.17	NIL	47.20±0.61	NIL	61.56±0.81	>400
Control	Chymostatin	8.24±0.11	Eserine	0.04±0.0001	Eserine	0.85±0.0001



Compd. No.	-R	Compd. No.	-R
6a		8c	
6b		8d	
6c		8e	
8a		8f	
8b		8g	

Scheme-1: Synthesis of carbamates 6a-c and 8a-g

in the molecule. In its ^{1}H-NMR spectrum, the signals appearing in aromatic region at 7.36-7.33 (m, 5H, H-2'- H-6') were assignable to the *O*-phenyl ring while two *ortho* coupled doublets appearing at δ 7.18 (d, *J* = 9.0 Hz, 2H, H-2, H-6) and 6.80 (d, *J* = 8.5 Hz, 2H, H-3, H-5) were characteristics of 1,4-disubstitued aromatic ring derived from metol. In the aliphatic region, a singlet appeared at δ 3.27 corresponding to the *N*-methyl group in the molecule. On the basis of these evidences the structure of 3 was assigned as *N*-(4-hydroxyphenyl)-*N*-methyl-*O*-phenyl carbamate. Similarly on the basis of structural evidences from ^{1}H-NMR, the structures of other *O*-substituted derivatives were elucidated as described in structural characterization section. The screening of these derivatives against α-chymotrypsin, acetylcholinesterase, and butyryl cholinesterase enzymes revealed that these were inactive against α-chymotrypsin but displayed moderate inhibitory potential against acetylcholinesterase and butyrylcholinesterase as it was evident from their IC_{50} values (Table-1). Among these, *N*-[4-(4-chlorobenzoxy)phenyl]-*N*-methyl-*O*-phenyl carbamate (8e) was found to be better inhibitor of AChE having IC_{50} value of 150.41±0.08 µmoles/L, relative to eserine, a reference standard with IC_{50} values of 0.04±0.0001 against this enzyme. Comparatively, this better inhibitory potential might be attributed to the substitution of 4-chlorobenzyl group at 4-*O* position in the molecule. The carbamate *N*-(4-benzoxyphenyl)-*N*-methyl-*O*-phenyl carbamate (8b), owing to the substitution of benzyl group at 4-*O* position also showed moderate activity against AChE with IC_{50} value of 189.51±0.05. The substitution of 3-chlorobenzyl group in 8c rendered this molecule a good inhibitor of BChE with IC_{50} value of 109.32±0.17 µmoles/L, relative to the standard eserine having IC_{50} value of 0.85±0.0001 µmoles/L. Afterwards, the low IC_{50} value of 8a (127.31±0.14 µmoles/L) explored that the substitution of ethyl group was also a good entity for inhibition of this enzyme. However, other substitutions retarded the inhibitory potential of these carbamates against this enzyme. Against α-chymotrypsin enzyme, only *N*-(4-benzoyloxyphenyl)-*N*-methyl-*O*-phenyl carbamate (6b) exhibited temperate inhibitory potential while all others were inactive.

CONCLUSION

The anticipated structures of the targeted carbamates are thoroughly supported by their spectroscopic data. From the enzyme inhibition screening data (Table-1), it might be generally concluded that some of the synthesized carbamates could be used as moderate inhibitors of cholinesterases and these are also ideally suited for further structural modification to obtain more potent and less cytotoxic therapeutic agents for the treatment of Alzheimer's disease.

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