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Synthesis and Evaluation of New Tacrine Analogues as Anti-Alzheimer's Agents

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Abstract

A Series of new tacrine analogues were designed, synthesized, characterized by respective spectral data and evaluated for cholinesterase inhibitory activity to be useful in Alzheimer's disease. Most of the synthesized compounds showed good *in vitro* inhibitory activities towards acetyl cholinesterase (AChE) and butyryl cholinesterase (BuChE) enzymes. Out of five compounds, synthesized, three (**6a, 6b, 6c**,) were evaluated for AChE and BuChE inhibitory activities,indicates that all the three compounds have exhibited almost similar potency against AChE with IC_{50} values between 0.31±0.02 and 0.37±0.04 µM, when compared to 0.23±0.01µM shown by the standard tacrine and 0.17µM exhibited by rivastigmine. Compound **6a** and **6b** with cyclopentano and cyclohexano fused tacrine analogs have shown almost equal potency to that of tacrine with IC_{50} values 0.34 and 0.31µM against AChE.

Keywords

Alzheimer's disease, antioxidants, anti-inflammatory, cholinesterase inhibitor, multi targeted ligands.

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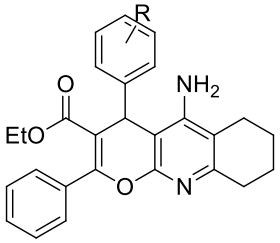
1.0. INTRODUCTION:

Alzheimer's disease (AD) is also known as senile dementia of the Alzheimer's type (SDAT), a type of brain disease that affects a huge number of elderly people. It is a progressive neurological illness characterized by early memory deterioration, as well as other symptoms such as language difficulties, visuospatial abnormalities, and loss of cognition, which worsen over time and eventually lead to death. Following cardiovascular disease, cancer, and cerebrovascular illness, Alzheimer's disease is now the fourth the largest cause of death in Western countries (Goedert et al., 2006). According to the World Health Organization (WHO), Alzheimer's disease (AD) will be more common in the next century than AIDS, cancer, and cardiovascular disease (Wimo et al., 2015). In the United States, over 17% of adults over the age of 75 suffer from Alzheimer's disease, with a projected cost of USD 236 billion for treatment, which may rise to over USD 700 billion by 2050 (Andreeva et al., 2017). Although the etiology of AD has not been fully explained, several factors, including amyloid- (A) deposits, tau protein aggregation, the exitotoxity hypothesis, oxidative stress (Jucker et al., 2011), and decreased acetylcholine levels (Sultana et al., 2010), have been implicated in the pathomechanism of AD. It has also been proposed that oxidative damage to intracellular structures may decrease mitochondrial metabolism, which acts as a causal component of improper cellular function and, evidently, cell death, as well as trigger inflammatory symptoms (Kaur et al. 2019; Meng et al.2007; Wu et al. 2007). There is currently no complete therapy available to cure AD, and clinical treatments only have behavioral effects. Numerous medications have already been used to control brain Aβ levels, either by decreasing production or by increasing clearance; nevertheless,

such a strategy failed to improve the cognitive performance of AD patients in trials. Due to accompanying toxicities, recent medication discoveries in the AD segment targeting BACE (beta-site amyloid precursor protein cleaving enzyme) have been unable to perform up to expectations. Tacrine, donepezil, galantamine, and rivastigmine are examples of drugs that work by increasing acetylcholine (ACh) levels by inhibiting acetylcholinesterase (AChE), as well as memantine, an NMDA receptor antagonist. Tacrine was the first clinical candidate to be introduced into the market for the treatment of Alzheimer's disease, but it was removed from the market due to significant hepatotoxicity caused by the free primary amine group.

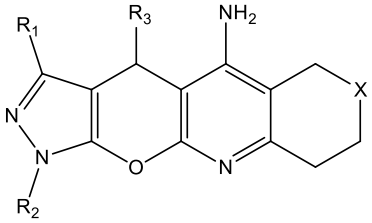
2.0 Rationale:

Literature survey reveals that various tacrine derivatives with substituted benzene ring having substitution at 4th position showed potent AChE inhibitory activity than tacrine molecule. Tacrine with unsubstituted amino group also showed potent activity against anticholinesterases and 15-LOX.



R= Cl, Br, OMe , F
IC_{50} = 0.069 and 1.35µM

Compounds with increase (X=CH_2-CH_2) or decrease (X=CH_2) ring size or by heteroatomic substitution (X=O, N, S) in place of cyclohexane ring also showed potent activity antiChEs and substitution at R_3 also has influence on activity.



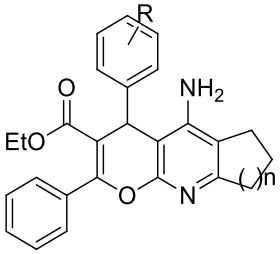
R_1=Ph, 2-Furyl

R_2=H,Ph

R_3=C_6H_5, 4-Me-C_6H_5, 4-NO_2-C_6H_5

Hence, it is proposed to synthesise tacrine derived pyranoquinoline carboxylates as shown in general structure I with benzene ring having substitution at 4th position of pyran ring and by substitution at cyclohexane ring of tacrine as shown in general structure **(a)** and evaluate them for Alzheimer's activity.

General Structure I



- n= 1, 2,
- R=H, Cl, Br.OMe, NO_2, F

3.0. Chemistry:

The melting points of open capillary tubes were determined using the VEEGO VMP-D Digital melting point equipment. The powdered compounds' FTIR spectra were recorded using KBr on a JASCO FTIR 4100 series spectrophotometer and are presented in cm-1. The 1H NMR and 13C NMR spectra were recorded on a BRUKER-II 400 (400 MHz NMR, 13C NMR 100 MHz) spectrophotometer with TMS as an internal reference. The compounds' purity was determined using pre-coated TLC plates, silica G as the stationary phase, iodine vapors, and ultraviolet light as the visualizing agent. Sigma-Aldrich, Hi Media, Bangalore, India, and others provided all chemicals, including conventional medications and solvents. The biochemical parameters were estimated using kits (Sigma-Aldrich).

3.1. Synthesis and Characterization:

3.1.1 Experimental work:

STEP-I:

Preparation of ethyl-6-amin-5-cyano-2,4-diphenyl-4H-pyran-3-carboxylate:

To the flask added ethyl-3-oxo-3-phenylpropanoate (**1**,1mmole), aromatic aldehyde (**2,**1mmole) Malononitrile (**3,**1mmole), piperidine (1 drop) in

ethanol (5ml) are refluxed for 3 hours. Progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was cooled to 0-5^{0}C, the precipitated solid was filtered and washed with cold ethanol and dried to give product [**4**].

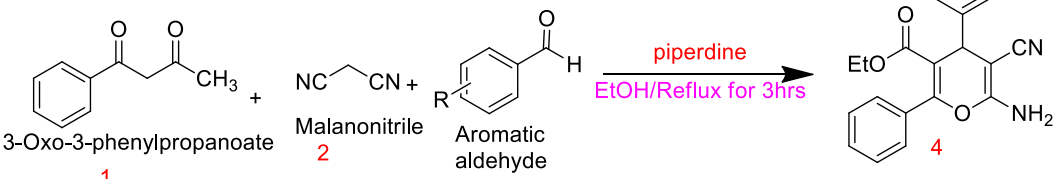
STEP-II:

Preparation of ethyl-5-amino-2, 4-diphenyl-6,7,8,9-tetrahydro-4H-pyran [2,3-b] quinoline- 3-carboxylate:

Into a flask aluminium chloride (1.5mmole) was suspended in dry 1,2-dichloroethane [5ml] at room temperature under nitrogen atmosphere. Stirred the mixture to get suspension for few minutes, then corresponding ethyl-6-amino-5-cyano-4H-pyran-3-carboxylate [1mmole, **4**], and cyclic ketone [**5**,1.5mmole] were added to suspension and reaction mixture was heated under reflux for 3-6 hrs. Progress of the reaction was monitored by TLC. After completion, water was added, and mixture was basified with 10% sodium hydroxide solution to P^H 8-9. The layers were separated, and aqueous layer was extracted with dichloromethane (3*15ml). The combined extracts were dried using sodium sulphate (Na_2SO_4) and concentrated. Purification by column chromatography on silica gel afforded the title compounds.

STEP-1:

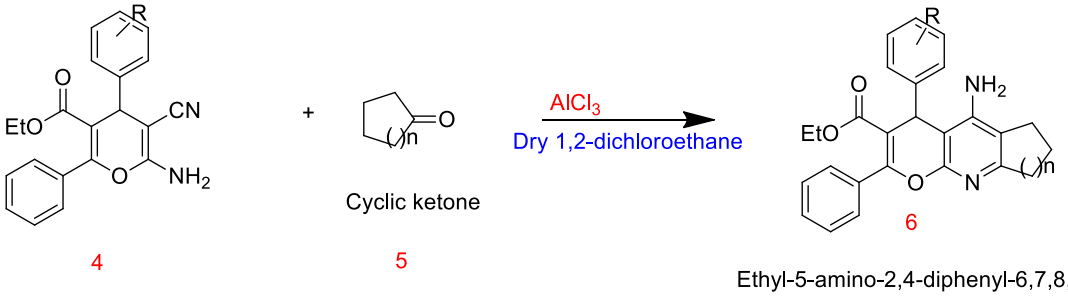
Preparation of Ethyl-6-amino-5-cyano-4H-pyran-3-carboxylate:



3-Oxo-3-phenylpropanoate (1) + Malanonitrile (2) + Aromatic aldehyde (3) → Ethyl-6-amino-5-cyano-4H-pyran-3-carboxylate (4)

STEP-2:

Ethyl-5-amino-2,4-diphenyl-6,7,8,9-tetrahydro-4H-pyrano[2,3-b] quinoline-3-carboxylate:



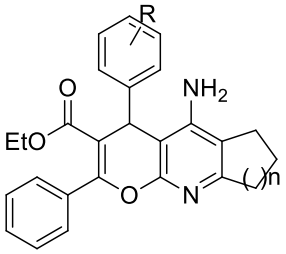
4 + Cyclic ketone (5) → Ethyl-5-amino-2,4-diphenyl-6,7,8,9-tetrahydro-4H-pyrano[2,3-b] quinoline-3-carboxylate (6)

n=1, 2,

R= H, OMe, Cl, Br, NO_2, F,

Scheme I

Table:1: Physical data of synthesized ethyl-5-amino-2, 4-diphenyl-6,7,8,9-tetrahydro-4H-pyran [2,3-b] quinoline- 3-carboxylate derivatives



n= 1, 2,

R= Cl, OCH_3, NO_2, Br

Hexane (7): Ethyl acetate (3) *

Compound	n	R	R_f *	Molecular formula	Mol. weight	MP (^{0}C)	%yield
6a	2	4-Cl	0.9	$C_{26}H_{23}ClN_2O_3$	460	200-204	45
6b	1	4-Cl	0.8	$C_{27}H_{25}ClN_2O_3$	446	201-210	48
6c	1	3,4,5-OCH_3	0.82	$C_{29}H_{30}N_2O_6$	502	190-192	49
6d	2	3,4,5-OCH_3	0.73	$C_{30}H_{32}N_2O_6$	516	190-194	48
6e	2	3,4-Cl	0.75	$C_{27}H_{24}Cl_2N_2O_3$	495	162-164	40

4.0. SPECTRAL DATA:

4.1.1. Ethyl 5-amino-4-(4-chlorophenyl)-2-phenyl-4,6,7,8-tetrahydrocyclopent [b]pyrano[3,2-e] pyridine-3-carboxylate (6a):

IR Spectral data (KBr, Cm^{-1}) : 3414 (N-H, str), 3056 (Aromatic ,C-H, str), 2932(Aliphatic, C-H, str), 1630(C=O, str), 1570(Aromatic, C=C, str), 1351(C-N, str), 1028(C-O, str), 750(C-Cl, str). **^{1}HNMR Spectral data** (400MHz, **DMSO**-d6): 1.26-1.30(t, 2H, Methyl, J=8.6 MHz), 1.80-1.91(m, 5H, Aliphatic), 2.70-2.73(t, 3H, Aliphatic, J=2 MHz), 2.70-2.78(t, 4H, Aliphatic, J=2.5 MHz), 3.90- 4.10(m, 2H, methylene), 5.06(s, 2H, Amine), 6.12(s, 1H, pyran ring), 7.20-7.22(d, 2H, P-Cholrophenyl C-2and C-6), 7.30-7.35(t, 2H, Phenyl C-3 and C-5), 7.48-7.50(d, 2H, Phenyl C-2and C-6), 7.56-7.59(t, 1H, Phenyl C-4,), 7.88-7.90(d, 2H, P-Chlorophenyl C-5 and C-3). **Mass spectrum** of this compound showed $[M+2]^+$ peak at 462.

4.1.2. Ethyl-5-amino-4-(4-Chlorophenyl)-2-phenyl-6, 7, 8, 9-tetrahydro-4H-pyrano [2, 3-b] quinoline-3-carboxylate (6b):

IR Spectra data (KBr, Cm^{-1}) ῡ : 3425 (N-H, str), 3080 (Aromatic ,C-H, str), 2923(Aliphatic, C-H, str), 1637 (C=O, str), 1572(Aromatic, C=C, str), 1353(C-N, str), 1022(C-O, str), 758(C-Cl, str). **^{1}HNMR Spectral data** (400MHz, **DMSO**-d6): 1.28-1.31(t, 3H, Methyl, J=6 MHz), 1.82-1.90(m, 4H, Aliphatic), 2.68-2.71(t, 2H, Aliphatic, J=2 MHz), 2.74-2.77(t, 2H, Aliphatic, J=2.5 MHz), 3.99- 4.16(m, 2H, methylene), 5.09(s, 2H, Amine), 6.19(s, 1H, pyran ring), 7.24-7.30(d, 2H, P-Cholrophenyl C-2and C-6), 7.31-7.42(t, 2H, Phenyl C-3 and C-5), 7.43-7.52(d, 2H, Phenyl C-2and C-6), 7.54-7.56(t, 1H, Phenyl C-4,), 7.87-7.89(d, 2H, P-Chlorophenyl C-5 and C-3).**Mass spectrum** of this compound showed $[M+2]^+$ peak at 448.

4.1.3.Ethyl-5-amino-2-phenyl-4-(3,4,5-trimethoxyphenyl)-4,6,7,8-tetra hydro cyclopenta [b] pyrano [3,2-e]pryridine-3-carboxylate (6c):

IR Spectra data (KBr, Cm^{-1}) ῡ **:** 3420(N-H, str), 2920(Aromatic, C-H str), 2851(Aliphatic, C-H str), 1640(C=O, str), 1545(C=C, str), 1380(C-N, str), 1111(C-O, str). **^{1}HNMR Spectral data** (400MHz, **DMSO**-d6): 1.20-1.23(t, 3H, Aliphatic, J=4.6 MHz), 2.40-2.48(m, 2H, Aliphatic), 3.08-3.11(t, 2H, Aliphatic, J=6 MHz), 3.40-3.44(q, 2H, Aliphatic,), 3.85-4.05(s, 9H, Methoxy), 4.06-4.16(m, 2H, methyl), 5.02(s, 2H, Amine), 6.10 (s, 1H, pyran ring), 6.45-6.46(s, 2H, Alkoxy phenyl $C_2$and C_6), 7.40-7.43(t, 2H, Phenyl C-3' and C-5') 7.50-7.53(t, 1H, Phenyl C-4'), 7.87-7.89(d, 2H, Phenyl C-2' and C-6'). **Mass spectrum** of this compound showed $[M+H]^{+\,peak}$ at 517.

4.1.4. Ethyl 5-amino-2-phenyl-4-(3,4,5-trimethoxyphenyl)-6,7,8,9-tetrahydro-4H-pyrano[2,3-b] quinoline-3-carboxylate (6d):

IR (KBr, Cm^{-1}) ῡ : 3438(N-H, str), 2923(Aromatic, C-H str), 2853(Aliphatic, C-H str), 1649(C=O, str), 1551(C=C, str), 1383(C-N, str), 1114(C-O, str). **^{1}HNMR Spectral data (400MHz, DMSO-d6):** 1.28-1.31(t, 3H, Aliphatic, J=6 MHz), 2.42-2.48(m, 2H, Aliphatic), 3.05-3.08(t, 2H, Aliphatic, J=6 MHz), 3.42-3.45(q, 2H, Aliphatic,), 3.85-4.05(s, 9H, Methoxy), 4.06-4.16(m, 2H, methyl), 5.09(s, 2H, Amine), 6.17(s, 1H, pyran ring), 6.45-6.46(s, 2H, Alkoxy phenyl $C_2$and C_6), 7.42-7.52(t, 2H, Phenyl C-3' and C-5') 7.54-7.56(t, 1H, Phenyl C-4'), 7.87-7.89(d, 2H, Phenyl C-2' and C-6'). **Mass spectrum** of this compound showed $[M+H]^+$ peak at 503.

4.1.5. Ethyl 5-amino-4-(3,4-dichlorophenyl)-2-phenyl-6,7,8,9-tetrahydro-4H-pyrano[2,3-b] quinoline-3-carboxylate

IR Spectral data (KBr, Cm^{-1}) : 3409 (N-H, str), 3064 (Aromatic ,C-H, str), 2901(Aliphatic, C-H, str), 1624 (C=O, str), 1542 (Aromatic, C=C, str), 1350(C-N, str), 1020(C-O, str), 715(C-Cl, str). **^{1}HNMR Spectral data** (400MHz, **DMSO**-d6): 1.29-1.32(t, 2H, Methyl, J=4.6 MHz), 1.90-1.95(m, 5H, Aliphatic), 2.65-2.68(t, 3H, Aliphatic, J=2 MHz), 2.74-2.78(t, 4H, Aliphatic, J=2.5 MHz), 3.98- 4.07(m, 2H, methylene), 5.01(s, 2H, Amine), 6.18(s, 1H, pyran ring), 7.24-7.26(d, 2H, P-Cholrophenyl C-2and C-6), 7.30-7.35(t, 2H, Phenyl C-3 and C-5), 7.48-7.50(d, 2H, Phenyl C-2and C-6), 7.57-7.61(t, 1H, Phenyl C-4,), 7.90-7.92(d, 2H, P-Chlorophenyl C-5 and C-3). **Mass spectrum** of this compound showed $[M+2]^+$ peak at 497.

5.0. PHARMACOLOGICAL EVALUATION:

5.1.1 *In Vivo* Evaluation of Anti-Alzheimer Activity

$AlCl_3$ induced memory impairment:

Drugs and chemicals:

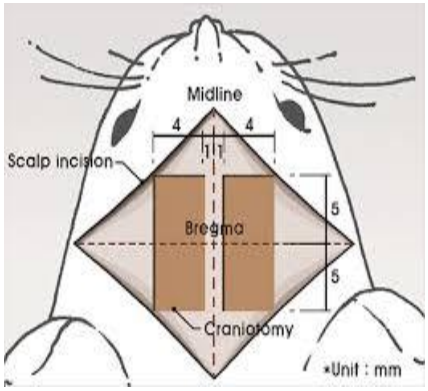
Aluminum chloride - hydrated ($AlCl_3.6H_2O$), was purchased from Sigma Chemical Co. (St. Louis, MO, USA). It was freshly dissolved in distilled water. All other chemicals and solvents were of highest grade-commercially available.

Experimental design :

Young Swiss-Albino mice (weighing about 20-30gm) were procured from the Madras Medical College animal house. The animals used for the entire study was approved by the Institutional Animal Ethical Committee which is certified by the Committee for the purpose of control and supervision of experiments on animals, India. Approved CPCSEA Registration No: 05/243/CPCSEA Dated: 10/08/2018.

5.1.2. Animals and induction of neurotoxicity:

Neurotoxicity was induced by intra cerebroventricular (i.c.v) injection of Aβ-peptide by identifying bregma point in the skull using stereotype apparatus (INCO, Arbala India). Each animal was injected with 10µl which 10µg of β -amyloid peptide (Laursen and Belknap, 1986).



Animals were divided into 5 groups of six animals each.

Group I: β-amyloid plaques (10µg/kg) were given I.P for 6 weeks after training.

Group II: Rivastigmine (200 mg/kg b.wt) was given I.P for 7 days after β-amyloid drug administration.

Group III: Rivastigmine (400 mg/kg b.wt) was given I.P for 7 days after β-amyloid drug administration.

Group IV: Test drug-I: (200mg/kg) was given I.P for 7 days after β-amyloid drug administration.

Group V: Test drug-II: (400mg/kg) was given I.P for 7 days after β-amyloid drug administration.

All treatments were administered at dose volume not exceeding 0.1ml/40g.

Behavioral tests (2 days' time interval between them) were carried out at the end of the six weeks; Morris Water Maze (MWM) test and Conditioned Avoidance (CA) test, Jumping box, rectangular maze.

5.2. BEHAVIOUR PARAMETERS ESTIMATION

5.2.1. JUMPING AVOIDANCE BOX (CONDITIONED AVOIDANCE TEST):

Box divided into 2 equal chambers by Plexiglas partition, with a gate providing access to adjacent compartment through 14*17 cm space. In each trial animal is subjected to light for 30 seconds followed by a sound stimulus for 10 seconds. Immediately after sound stimulus, mice receive a single low intensity foot shock (0.5mA, 3 sec).each animal received a daily session of 15 trials with an inter trial duration of 15 seconds for 5 days.

JUMPING BOX

5.2.2. RECTANGULAR MAZE TEST:

Assessment of memory was done using medicraft rectangular maze. The apparatus consisted of three interconnected chambers A,B&C. chamber B constituted the maze . Food deprived mice were placed in chamber A& challenged to learn & to remember the location of C, after travelling through chamber B. Their presence in chamber C was indicated by a pilot light. Chamber C contained the reward which was food for the hungry animal. The animals were trained which for consecutive daily sessions, & the time required to transverse the maze was noted. They were considered trained when the maze completion time for three consecutive days were more (or) less constant .Maze traversing time was than recorded for each animal before & after drug treatment.

RECTANGULAR MAZE

5.2.3. Y-MAZE TEST:

Y-maze served as the enteroceptive model to evaluate acquisition of spatial memory in experimental animal models. The apparatus was constructed of plain wood & consist of identical three arms. The arms were randomly designated.

A. Start arm in which the rat started to explore (always open)

B. Novel arm: which was blocked during the first trial, but open during the second trial and the other arm was always open.

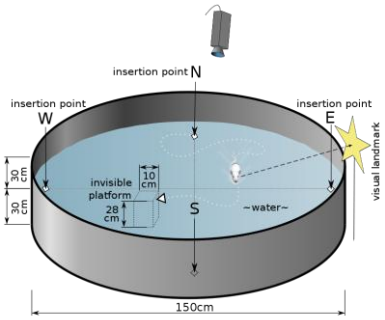
Each arm was 35cm×6cm×15cm (width × height × length). The maze has an equilateral centre, each arm of the Y beginning from each side of the triangle & extending radially away from the centre at an angle of 120°, forming the letter Y shape of the maze. It was important that the three arms be made similar to prevent preference on the part of the animal when introduced into the maze. The floor of the maze was covered with sawdust, which was mixed after each individual trial in order to eliminate olfactory stimuli. At the start of each session, each animal was gently placed at the end of the start arm, facing away from the central platform. The time taken by the animal to move from the end of the arm to the centre of the maze was measured & recording as the Transfer Latency (TL). TL was recorded for each animal & if a mice did not get to the centre of the maze within 90 sec, it was gently pushed to the centre & TL was assigned 90sec. TL was recorded for each animal in each trial & expressed as inflexion ratio (IR). IR was calculated by the formula described as

INFLEXION RATIO = (L1-L0)/L0

Where L1 is the initial TL (sec) & L0 is the TL (sec) after 24hr

Morris Water Maze:

The Morris water maze was developed by Richard Morris in 1984. It is the most popular task in behavioral neuroscience and in its most basic form; it assesses spatial learning and memory along with no spatial discrimination learning. Performance in the Morris Water Maze is acutely sensitive to manipulations of the hippocampus. In this model the animals are placed into a large circular pool of water, and they can escape on to a hidden platform. The platform is hidden by its placement just below the water surface which is made opaque by mixing Titanium dioxide in the water. Therefore, the platform offers no local cues to guide the escape behavior. The animal can escape from swimming by climbing on to the platform and with time the animal apparently learns the spatial location of the platform from any starting position at the circumference of the pool. Morris water maze consists of a large circular tank made of black opaque polyvinyl chloride or hardboard coated with fiber glass and resin and then surface painted white (1.8-2m in diameter and 0.4-0.6m high). The pool is filled up to a height of 30cm with water until the top of the platform is submerged 1cm below the water surface and maintained at around 25 °C and rendered opaque by addition of small quantity of milk or nontoxic white colour. The pool is provided with filling and draining facilities and is mounted at waist level. The tank is hypothetically divided into 4 equal quadrants the platform (11cm2) of 29 cm height is in the centre of one of these 4 quadrants. Before beginning acquisition training, mice are given a pertaining acclimatization session during which they are allowed to swim in the pool for 5min without the platform.



The platform remains fixed in the position during the training session. Each animal is subjected to four consecutive trials for four days during which they are allowed to escape on the hidden platform and allowed to remain for 20seconds. Mice are released from the four points along the perimeter of the maze arbitrarily designated as N, S, W and E. Escape latency time to locate the hidden platform in water maze is noted as an index of acquisition or learning. In case the animal is unable to locate the hidden platform within 120seconds, it is gently guided by hand to the platform and allowed to remain there for 20seconds. In each training session, the escape latency was recorded. After 4 days trail and 6 weeks treatment the animals were treated with amnesia inducing agent, 30 min after last IP dose of test compounds. After 1 hour mice were allowed to swim and the escape latency were recorded.

5.3. Estimation of Brain Cholinesterase:

Acetyl cholinesterase enzyme activity was estimated by Ellman's method.

Reagents

5.3.1. 0.1M phosphate buffer

- Solution A: 5.22gm of potassium hydrogen phosphate and 4.68gm of sodium hydrogen phosphate are dissolved in 150ml of distilled water.
- Solution B: 6.2gm of sodium hydroxide dissolved in 150ml of distilled water. Solution B is added to solution A to get the deserved pH (8.0 or 7.0) and then finally the volume is made upto 300ml with distilled water.

5.3.2. DTNB Reagent

39.6mg of DTNB with 15mg of sodium bicarbonate is dissolved in 10ml of 0.1M phosphate buffer (pH 7.0)

5.3.3. Acetylthiocholine (ATC)

21.67mg of acetylthiocholine was dissolved in 1ml of distilled water.

5.3.4. *In Vitro* Evaluation of Anti-Alzheimer Activity:

Acetyl Cholinesterase also known as RBC cholinesterase is found primarily in the blood and neural synapses. Low serum cholinesterase activity may relate to exposure to insecticides or to one of a number of variant genotypes. AChE catalyzes the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid, a reaction necessary to allow a cholinergic neuron to return to its resting state after activation. Cholinesterase levels of cells and plasma are used as a guide in establishing safety precautions relative to exposure and contact, as well as a guide in determining the need for workers to be removed from areas of contact with the organic phosphate insecticides. Simple, direct and automation-ready procedures for measuring AChE activity are very desirable. Acetylcholinesterase Assay Kit is based on an improved Ellman method, in which thiocholine produced by the action of acetylcholinesterase forms a yellow color with 5,5'-dithiobis (2-nitrobenzoic acid). The intensity of the product color, measured at 412 nm, is proportionate to the enzyme activity in the sample.

Materials Supplied
List of components:

Component	Amount
Assay Buffer: (pH 7.5	30mL
Reagent	240mg
Calibrater (equivalent to 200 U/L)	4mL

Storage Instruction

Store all reagents at room temperature. Shelf life: 6 months.

Materials Required but Not Supplied

Pipetting (multi-channel) devices. Clear-bottom 96 - well plates (e.g. Corning CoStar) and plate reader.

Precautions for Use

• Precautions

1. Reagents are for research use only. Normal precautions for laboratory reagents should be exercised while using the reagents.
2. This assay is based on an enzyme-catalyzed kinetic reaction. The addition of a Working Reagent should be quick and mixing should be brief but thorough. Use of a multi-channel pipette is recommended. For assays in standard 1 mL cuvette, use 1 mL water and 1 mL Calibrator, 50 µL sample + 950 µL Working Reagent.

1. Reagent Preparation

The Working Reagent should be prepared freshly and used within 30 min. Each reaction requires 2 mg of reagent. Calculate the amount of reagent needed and weigh this amount (mg) in a centrifuge tube. Add 200 µL Assay Buffer per 2 mg reagent. Vortex to dissolve.

2. Sample Preparation

Blood samples should be diluted 40-fold in the Assay Buffer, e.g. accurately pipette 5 µL blood and mix thoroughly with 195 µL Assay Buffer. Tissue or cell lysates are prepared by brief sonication or homogenization in 0.1M phosphate buffer (pH 7.5), followed by centrifugation at 14,000 rpm for 5 min. Use supernatant for assay. Ideally samples should be assayed fresh. If this is not possible, refrigerate samples and assay them within 24hours.

3. Assay Procedure

Calibrator: transfer 200 µL water and 200 µL calibrator separately into wells of a clear bottom 96-well plate. Samples: add 10 µL sample per well in separate wells. Reaction: transfer 190µL freshly prepared Working Reagent to all sample wells and tap plate briefly to mix. Read OD412nm at 2 min and at 10 min in a plate reader.

6.0. RESULTS AND DISCUSSIONS:

6.1.1 Results of the behavioral parameters: indicate that there is a considerable decrease in time required to traverse the maze in dose dependent manner in animal treated with test compounds (200mg/ml and 400 mg/ml). For instance, in rectangular maze test and Y-maze test, in case of compound **6a** at a dose of 200mg/kg, the time required 145.6 sec before treatment and 135.8 sec after treatment with a decrease of 9.8 sec. At 400mg dose, the time required to traverse was reduced by 27 sec, indicating dose dependent improvement in traversing the maze. Similar improvements were observed in case of compound **6b** and **6c**. However, the time required to traverse the maze in case of the standard was much less i.e., 24 sec and 35 sec respectively at 200 and 400 mg/ml (**Table 2**).

Table:2 Results of the behavioural parameters

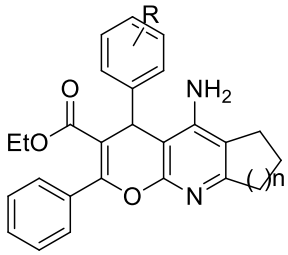
Sample	Dose (mg/ml)	Y maze test (Sec)		Rectangular maze test (Sec)		Jumping box test (Sec)	
		Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
6a	200	55.16±3.15	48.61±3.15	145.6±2.37	135.83±2.37	28.6±0.22	21.5±0.22
	400	57.25±3.15	41.73±3.15	151.7±2.37	124.75±2.37	29.5±0.22	18.7±0.22
6b	200	59.60±2.87	52.57±2.87	150.54±10.05	141.67±10.05	26.8±2.07	24.6±2.07
	400	58.79±2.87	45.57±2.87	158.54±10.05	135.56±10.05	30.6±2.07	21.3±2.07
6c	200	56.97±2.98	48.93±2.98	140.62±2.15	128.66±2.15	28.09±1.21	24.69±1.21
	400	59.42±2.98	41.02±2.98	138.73±2.15	120.05±2.15	28.20±1.21	20.70±1.21
Tacrine	200	54.02±2.06	34.11±2.06	142.23±2.21	118.16±2.21	29.07±0.71	18.57±0.71
	400	58.63±2.06	30.32±2.06	145.42±2.21	110.61±2.21	28.32±0.71	12.30±0.71
Control		41.19±2.18		76.87±2.53		14±1.09	

6.1.2. Results of the Invitro AChE and BuChE inhibitory activities:

Out of five compounds, synthesized, three (**6a, 6b, 6c**,) were evaluated for AChE and BuChE inhibitory activities, perusal of **Table 2** indicates that all the three compounds have exhibited almost similar potency against AChE with IC_{50} values between 0.31±0.02 and 0.37±0.04 µM, when compared to 0.23±0.01µM shown by the standard tacrine and 0.17µM exhibited by rivastigmine. Compound **6a** and **6b** with cyclopentano and cyclohexano fused tacrine analogs have shown almost equal potency to that of tacrine with IC_{50} values 0.34 and 0.31µM against AChE. In compound **6b** replacement of 4-chloro group with 3, 4, 5-trimethoxy groups resulted in a little decrease in potency (IC50=0.37±0.04µM). Compound **6a** (cyclohexane analog) with 4-chlorophenyl substituent showed greater potency against BuChE with IC_{50} of 4.5±0.5µM than the corresponding cyclopentano analog **6b**, with an IC_{50} of 5.5±1.4µM. In BuChE inhibition assay too the compound **6c** showed the least potency with IC_{50} of

6.8±0.5µM. However, all the three compounds were found to be less potent than the both the standards Tacrine and Rivastigmine in BuChE inhibition assay. Compound **6b** and **6c** showed best selectivity towards AChE as per the calculations BChE/AChE selectivity. Best selectivity **(18)** is related to the most active anti-AChE compound (**6b** and **6c**).Slective index is less i,e **(13)** for the compound **6a.** Thus, Tacrine derived pyranoquinolines and their analogs have showed significant potency against AChE without much change in the potency with change in size of "D" ring (cyclopentane and cyclohexane). Further, it is concluded that the replacement of electron withdrawing chlorine with electron releasing methoxy groups did not result in greater diference in the anti-AChE potency.

Table 3: Assay of Acetylcholine esterase inhibiton and Butrylcholine esterase inhibition. (IC_{50}) values of synthesized tacrine derivatives.



General Structure-I

S.No	Compounds	*IC_{50}* against AChE (µM)	*IC_{50}* against BChE (µM)	Selectivity index (BChE/AChE)
1	6a	0.34±0.01	4.5±0.5	13.20
2	6b	0.31±0.02	5.5±0.3	18.03
3	6c	0.37±0.04	6.8±0.5	18.37
4	Tacrine	0.230±0.01	0.78±0.01	3.39
5	Rivastigmine	0.17±0.3	0.64±0.02	3.70
6	Negative control β-Amyloid	0.78±0.03	8.35±0.12	10.70

Inhibitor concentration (Mean ± SD of three experiments) required for50% inactivation of AChE and BChE. Selectivity index: IC_{50} BChE / IC_{50} AChE .

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Conflict of Interest:
The authors declare that they have no conflict of interest.

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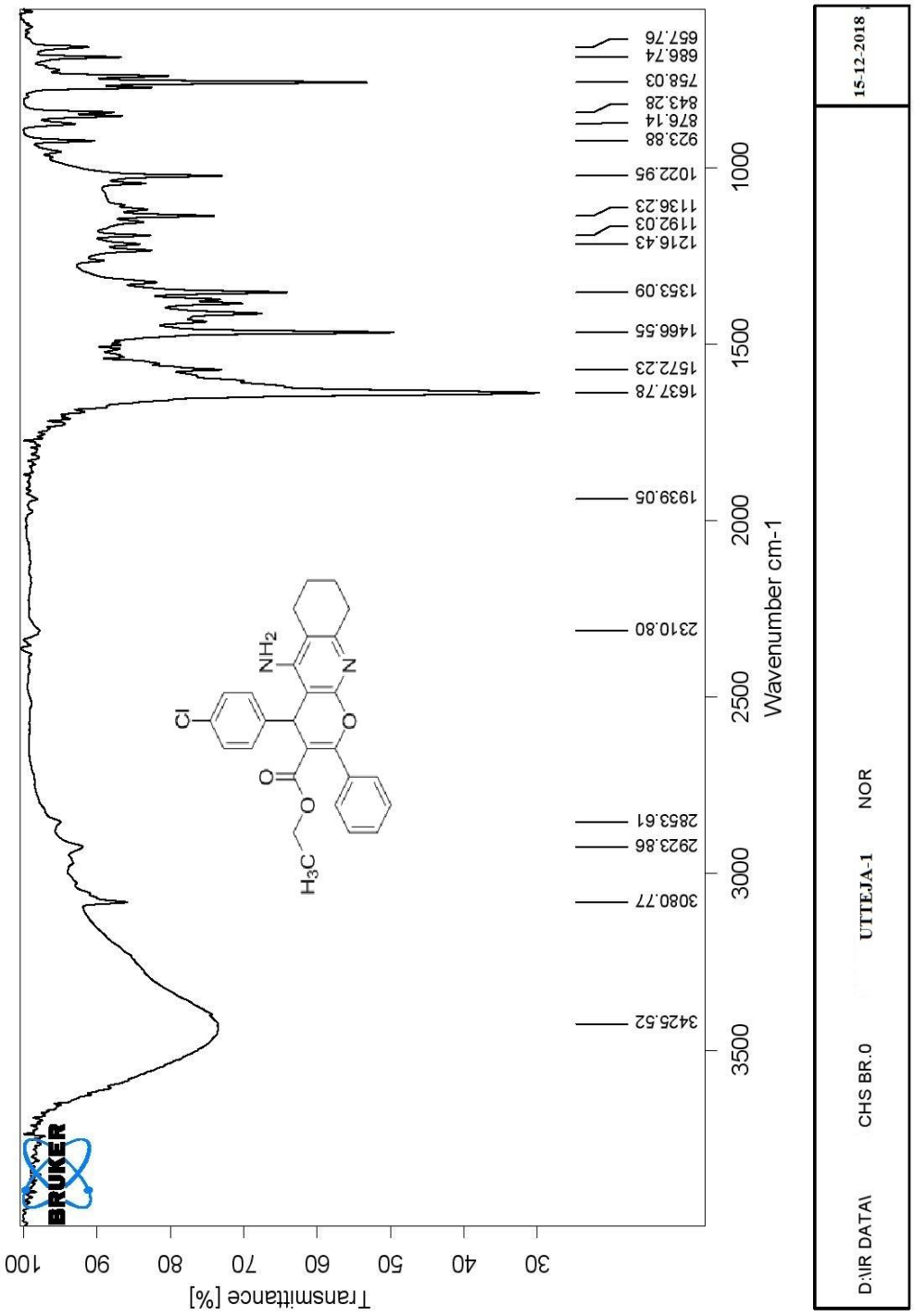
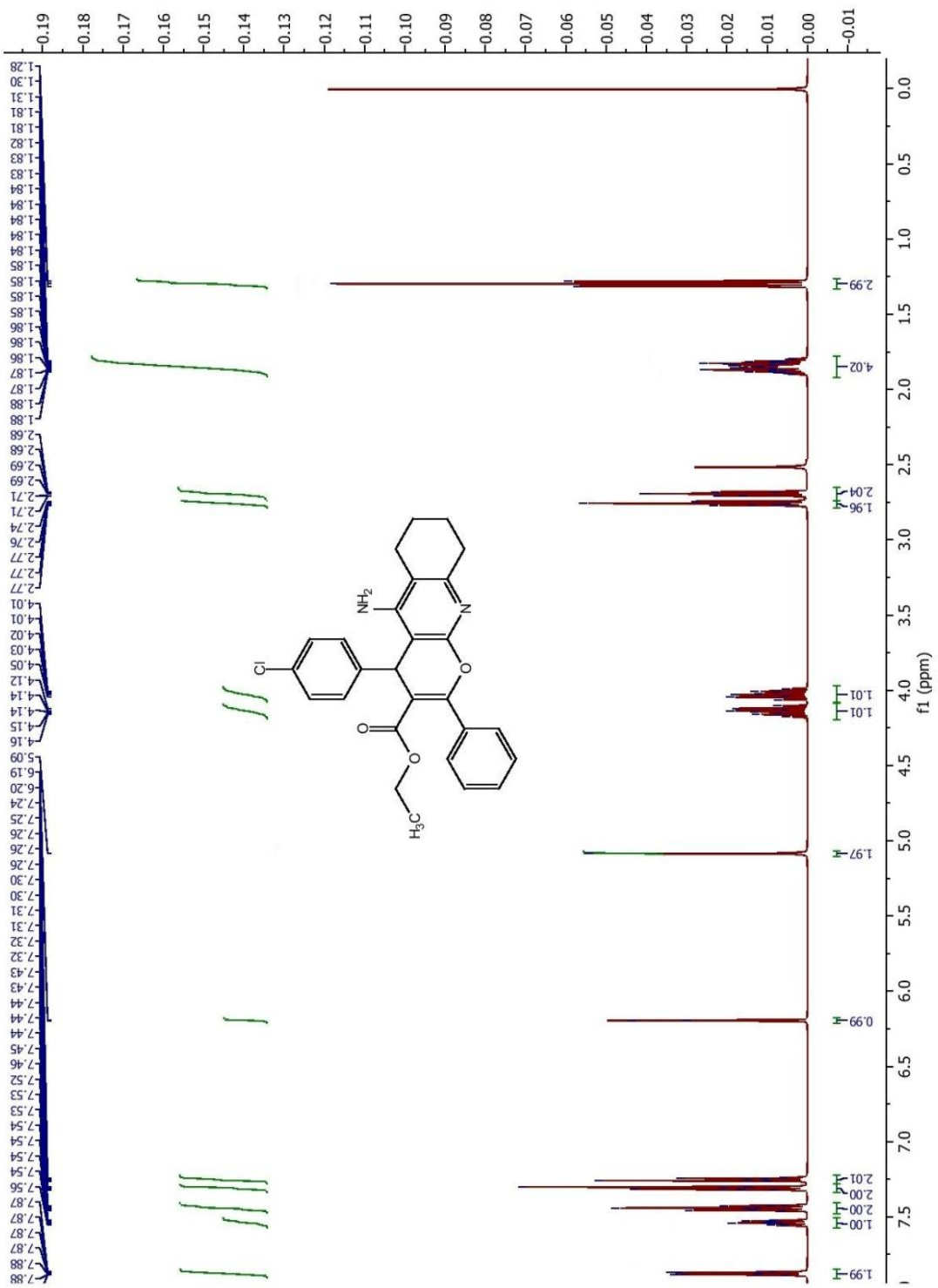


IR Spectrum of Ethyl-5-*amino*-4-(4-Chlorophenyl)-2-phenyl-6, 7, 8, 9-tetrahydro-4H-pyrano [2, 3-b] *quinoline*-3-carboxylate (6a)



^{1}H-NMR spectra of *Ethyl-5-amino-4-(4-Chlorophenyl)-2-phenyl-6, 7, 8, 9-tetrahydro-4H-pyrano [2, 3-b] quinoline-3-carboxylate (6a)*

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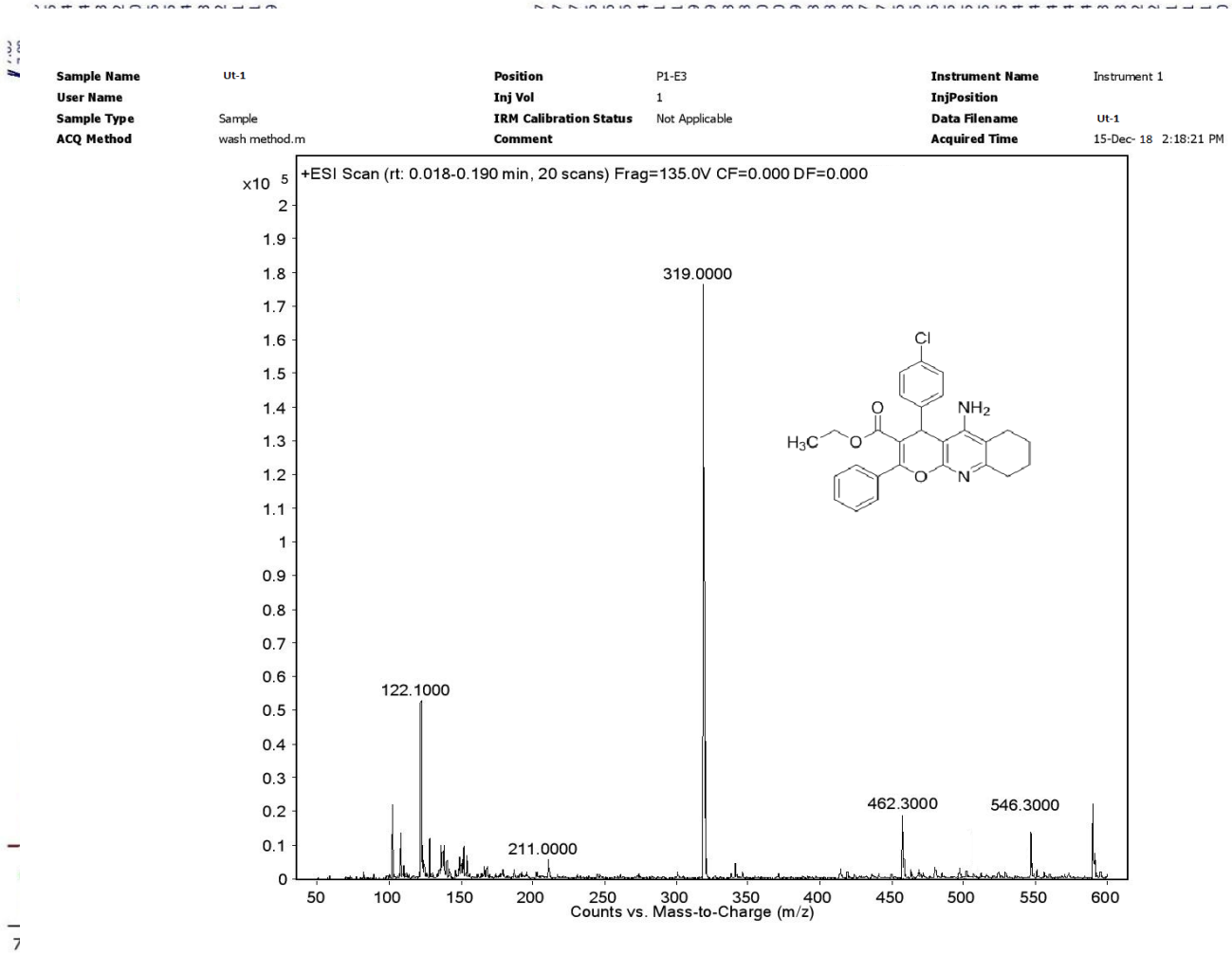
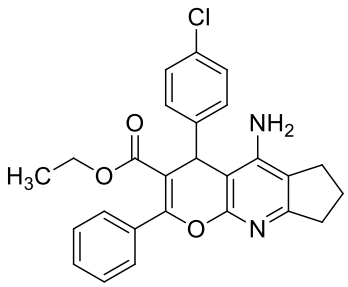


Mass Spectrum of *Ethyl-5-amino-4-(4-Chlorophenyl)-2-phenyl-6, 7, 8, 9-tetrahydro-4H-pyrano [2, 3-b] quinoline-3-carboxylate (6a)*

1b)

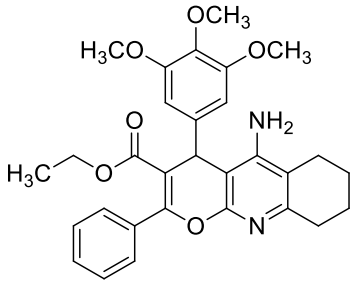


Ethyl 5-amino-4-(4-chlorophenyl)-2-phenyl-4,6,7,8-tetrahydrocyclopenta[*b*]pyrano[3,2-*e*]pyridine-3-carboxylate

Mol.Formula: $C_{26}H_{23}ClN_2O_3$ **Melting Point:** 201-210°C

Yield: 48% **R_f :** 0.8

1d)

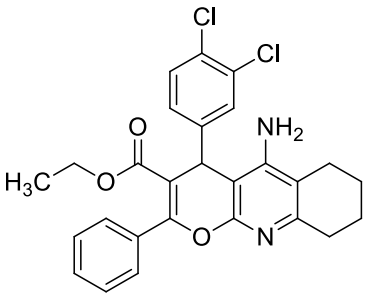


Ethyl 5-amino-2-phenyl-4-(3,4,5-trimethoxyphenyl)-6,7,8,9-tetrahydro-4*H*-pyrano[2,3-*b*]quinoline-3-carboxylate

Mol.Formula: $C_{30}H_{32}N_2O_6$ **Melting Point:** 190-194°C

Yield: 48% **R_f :** 0.73

1e)



Ethyl 5-amino-4-(3,4-dichlorophenyl)-2-phenyl-6,7,8,9-tetrahydro-4H-pyrano[2,3-b]quinoline-3-carboxylate

Mol.Formula: $C_{27}H_{24}Cl_2N_2O_3$ **Melting Point:** 162-164°C

Yield: 40% **R_f :** 0.75