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Research Article

SYNTHESIS AND CHARACTERIZATION OF 4-METHYLCOUMARIN DERIVATIVES AS ANTI-CHOLINESTERASE INHIBITORS

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ABSTRACT
The objective of the present work was to synthesize 4-methylcoumarin derivatives and evaluate anti-cholinesterase activity (in vitro) of the compounds. 4-Methyl coumarin derivatives was synthesized using the Pechmann synthesis using resorcinol, and ethylacetoacetate. The anti-cholinesterase activity of the synthesized compounds was studied by modified Ellman's method and the percent inhibition and IC50 was calculated. The yield of the compounds was in the range of 65-69%. All the compounds were soluble in chloroform and DMSO whereas insoluble in water. The synthesized derivatives were able to inhibit AChE activity dose dependently form 21.98 to 70.93%. The IC50 was calculated from inhibition percentage and was found to be 97.34 mM, 8.16 mM, 5.67 mM, 13.21 mM and 12.42 mM for C1 to C5 respectively. The inhibition of cholinesterase enzyme prevents the breakdown of acetylcholine into its component's choline and acetyl CoA.

Keywords: Coumarin, anti-cholinesterase, Pechmann synthesis, Ellman's method, inhibitory concentration.

INTRODUCTION
Cholinesterase is a family of enzymes that catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh) into choline and acetic acid, a reaction necessary to allow a cholinergic neuron to return to its resting state after activation [1]. Acetylcholinesterase is involved in the termination of impulse transmission by rapid hydrolysis of the neurotransmitter acetylcholine in numerous cholinergic pathways in the central and peripheral nervous systems [2]. AChE inhibitors or anti-cholinesterases inhibit the cholinesterase enzyme from breaking down ACh, increasing both the level and duration of the neurotransmitter action [3]. The enzyme inactivation, induced by various inhibitors, leads to acetylcholine accumulation, hyperstimulation of nicotinic and muscarinic receptors, and disrupted neurotransmission. Hence, acetylcholinesterase inhibitors, interacting with the enzyme as their primary target, are applied as relevant drugs and toxins.
Coumarin is a colourless crystalline solid with a sweet odour resembling the scent of vanilla and a bitter taste [4]. It is found in many plants, where it may serve as a chemical Défense against predators [5]. Methyl coumarin have been widely investigated for various pharmacological actions including cardiovascular effects, antihistaminic, antibacterial etc [6-10]. Previous studies have also related coumarin nucleus to be effective in several disease conditions and also effective against cholinesterase as anti-Alzheimer. In the present investigation we planned to design and synthesize novel 4-methylcoumarin derivatives for anti-cholinesterase activity.
Material and Methods
All chemical, solvent and reagents were used as obtained; melting point were assessed in open capillary and solubility was qualitatively analysed. TLC was done on precoated silica gel plates to monitor reaction completion.
The steps adopted in the synthesis of the 4-methycoumarin derivatives (Scheme 1) are depicted in the scheme below which is adopted from the literature reported [11] and modified as per requirement.

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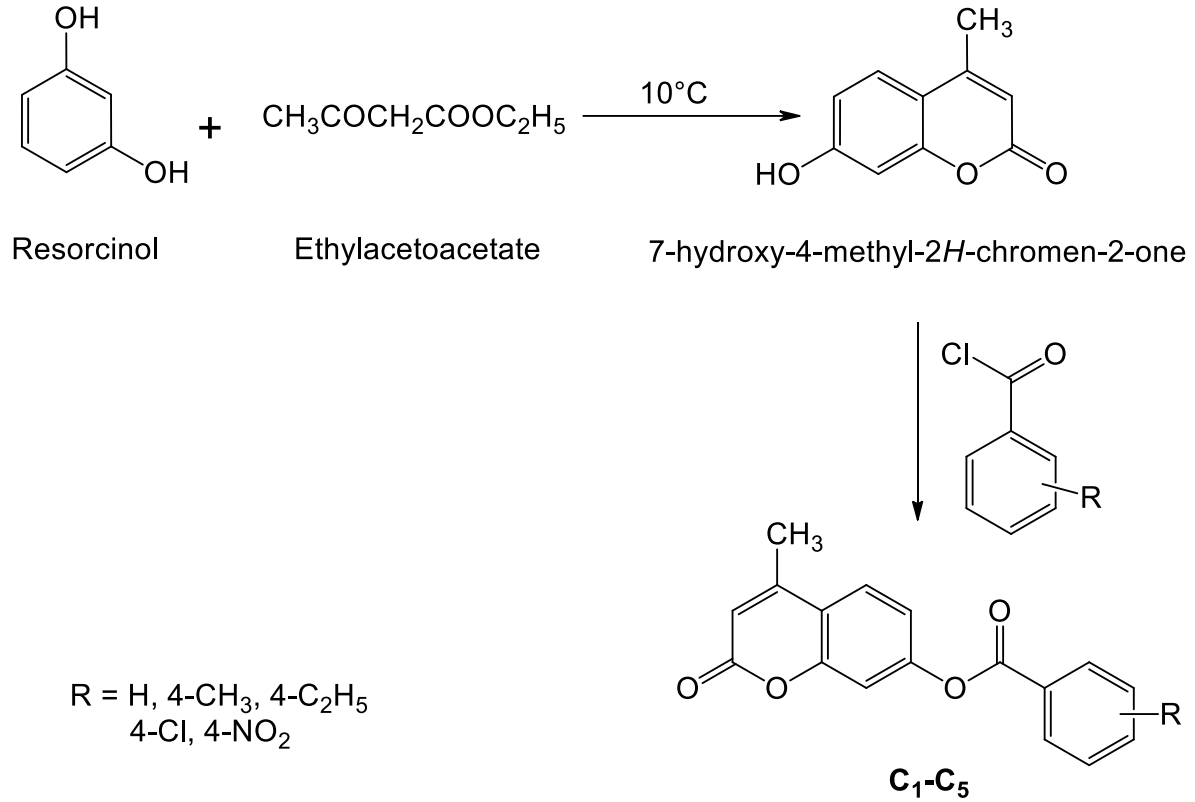


Scheme 1. Pathway to synthesized coumarin derivatives

Step 1. Synthesis of 4-methyl-7-hydroxycoumarin

1 gm of resorcinol was added to 1.18 ml ethyl acetoacetate and this mixture was cooled to 10°C. 7 ml conc. H2SO4 was added with constant stirring for 12hr. reaction mixture was poured in crushed ice. The solid product obtained was dissolve in 5% NaOH and then acidified to obtained brown solid of 4-methyl-7-hydroxycoumarin.

Step 2. General method for synthesis of target coumarin derivatives (C1-C5)

The mixture of 7-hydroxy-4-methyl-2H-chromen-2-one (0.001 mo) and appropriate benzoyl chloride derivative (0.001 mol) were stirred for 1 hr. The reaction mixture was poured in crushed ice, to get the 4-methyl-2-oxo-2H-chromen-7-yl benzoate. It is crystallized from ethanol.

Evaluation of Anti-cholinesterase Activity

The synthesized compounds were individually dissolved in required volume of dimethylsulfoxide (DMSO) and diluted using phosphate buffer solution (PBS) (pH = 7.8) for the final range of concentrations (2-10 µM).

A common chromogenic agent, Ellman's reagent was used for AChE catalyzed hydrolysis. In fresh 96-well microplates, each well was filled with 40 µL of 50 mM Tris-HCl buffer pH 8.0, 20 µL of test sample solution, 100 µL of Ellman's reagent (prepared by dissolving 18.5 mg reagent in 10 mL of buffer), and 20 µL of AChE solution (prepared by dissolving 0.26 U/mL enzyme in 15 mL buffer). The contents were mixed and incubated at 25°C for 15 min. The absorbance was measured at 412 nm. Now, 20 µL of 15 mM acetyl thiocholine (ACTh) (prepared by dissolving in water) was added as a substrate to the wells and the plate was incubated for 20 min at 37°C. Blank determination was done without test samples to obtain 100% AChE activity. The absorbance was measured at 412 nm and the percentage AChE inhibition was calculated using formula [12,13]:

$\%AChE\ inhibition = (A_{blank}-A_{sample})/A_{blank} *100$

RESULTS AND DISCUSSION

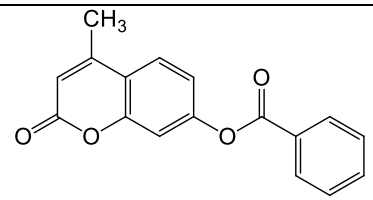
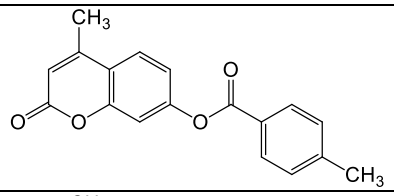
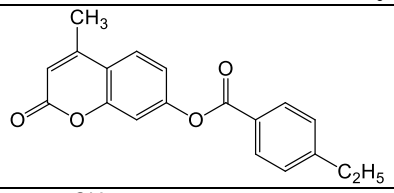
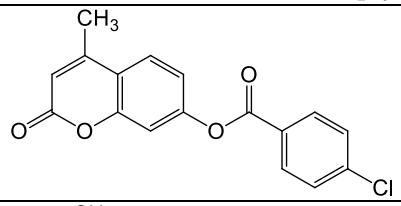
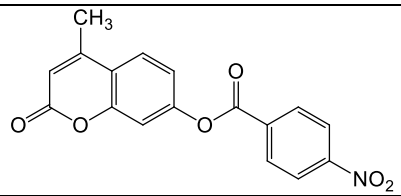
RESULTS

Chemistry

The target molecule C1-C5 was synthesized in two steps as per scheme 1.

The structure, yield, color, retention factor and melting point of all the synthesized compounds are depicted in Table 1.

Table 1. Physical Parameters of the synthesized compounds

Compound	Structure	Yield (%)	Color	Melting Point (°C)	Retention factor (R_f value)
C_1		68	Brown	198-200	0.72
C_2		69	Dark Yellow	208-210	0.65
C_3		65	Brown	211-213	0.69
C_4		67	Dark Yellow	175-177	0.61
C_5		65	Brown	171-173	0.59

The solubility characteristics (qualitative) of the synthesized compounds is various solvents was assessed. The results reveal the all the compounds were soluble in water and DMSO easily.

The spectral studies (NMR, Mass and IR) were conducted to confirm the structure of the synthesized compounds. The spectra were obtained for the samples and the interpretation of each spectrum was carried out to ascertain the formation of desired bonds and incorporation of the functional groups (Table 2).

Table 2. Spectral interpretation of C_1-C_5

Compound	IR peaks	NMR Signals	Mass Peaks
4-methyl-2-oxo-2H-chromen-7-yl benzoate, C_1	1657.45 (C=O)	2.44 (H, CH_3), 6.09-8.15 (H, aromatic)	280.4 (M^+)
4-methyl-2-oxo-2H-chromen-7-yl 4-methylbenzoate, C_2	1646.57 (C=O)	2.44 (H, CH_3), 6.09-8.18 (H, aromatic)	294.3 (M^+)
4-methyl-2-oxo-2H-chromen-7-yl 4-ethylbenzoate, C_3	1654.58 (C=O)	2.44 (H, CH_3), 2.69 (H, CH_3), 1.22 (H, CH_2), 6.09-7.96 (H, aromatic)	308.3 (M^++1)
4-methyl-2-oxo-2H-chromen-7-yl 4-chlorobenzoate, C_4	1654.66 (C=O), 818.83 (C-Cl)	2.44 (H, CH_3), 6.09-7.98 (H, aromatic)	315.2 (M^++1)
4-methyl-2-oxo-2H-chromen-7-yl 4-nitrobenzoate, C_5	1665.70 (C=O), 1504.23 (C-NO_2)	2.44 (H, CH_3), 6.09-8.40 (H, aromatic)	325.3 (M^+)

Anticholinesterase activity

The anticholinesterase activity was evaluated by Ellman's method and the results of inhibition of cholinesterase activity was calculated from the absorbance of blank and the test solutions (Table 3).

Table 3. %Inhibition of cholinesterase action

	% AChE inhibition				
Code	**2 mM**	**4 mM**	**6 mM**	**8 mM**	**10 mM**
C_1	29.44	39.38	45.88	51.81	59.27
C_2	28.48	35.18	42.44	48.75	56.97
C_3	31.93	43.21	49.90	61.95	70.93
C_4	21.98	26.76	31.73	36.52	42.25
C_5	23.70	27.91	34.03	39.19	43.40

The synthesized derivatives were able to inhibit AChE activity dose dependently form 3.54 to 64.06%. The IC50 was calculated from inhibition percentage and was found to be 7.34 mM, 8.16 mM, 5.67 mM, 13.21 mM and 12.42 mM for C1 to C5 respectively.

Discussion

Chemistry

The synthesis of 4-methylcoumarin from □-keto esters follows the basics of pechmann reaction. It involves the condensation of ß-ketoester with ethylacetoacetate in the presence of a catalyst such as sulfuric acid. The reaction is typically carried out at low temperatures (usually 10°C or less) to prevent the side reactions and keep a control on the reaction. The mechanism of this condensation is a three step procedure involving transesterification in the first step. Here the acid catalyzed conversion of the ethylacetoacetate is occurs, activating the carbonyl group. This is the key step responsible to generate the reactive species. In the second step, electrophilic aromatic substitution occurs where the activated carbonyl acts as the electrophile and attacks the phenol at position ortho to the hydroxyl group leading to the formation of a new carbon-carbon bond, producing an intermediate. In the last step of the reaction dehydration occurs where the cyclic intermediate loses water to create the stable coumarin ring system. The acidic medium helps in cyclization and aromatization of the ring. The preparation of the target compounds from the 7-hydroxy-4-methyl coumarin occurs via a simple substitution on the hydroxyl group. The spectral characterization of the synthesized derivatives revealed the presence of aromatic protons, and the protons of methyl in all the compounds. The FTIR spectrum exhibited the stretching vibrations due to C-H, C-C, C=C, C-O and C=O in the compounds. Vibrations of C-Cl and C-N were also found to be present in compound C4 and C5 respectively.

Anticholinesterase activity

The inhibition of cholinesterase enzyme prevents the breakdown of acetylcholine into its components choline and acetyl CoA. The compounds were able to inhibit cholinesterase enzyme in a dose dependent manner. The results show that the presence of electron donating group in the compound was beneficial for anticholinesterase action (C2 & C3). Presence of strong electron withdrawing group in compound was detrimental for anticholinesterase action (C4 & C5). Non-substituted compound (C1) was more effective in inhibition of cholinesterase action than a compounds with electron withdrawing substituents.

CONCLUSION

In this work, coumarin derivatives were synthesized and evaluated for anticholinesterase action. Compound C1, C2 and C3 were able to produce anticholinesterase action on less than 10 mM concentration.

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