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Original article

Synthesis and biological evaluation of functionalized coumarins as acetylcholinesterase inhibitors

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Abstract

Three series of functionalized coumarin compounds were designed and prepared as cholinesterase (AChE and BuChE) inhibitors. The biological profile against AChE and BuChE of the prepared compounds was determined. Compound **7b** exhibited a mixed-type of AChE inhibitor with IC_{50} value for the AChE inhibition of 0.19 ± 0.01 μM and a high selectivity for AChE/BuChE, and compound **6b** acted as non-competitive AChE inhibitor with IC_{50} value of 0.43 ± 0.02 μM. Structure–activity relationships (SARs) of prepared compounds were discussed.

Keywords: Functionalized coumarins; Cholinesterase inhibitor; Synthesis

1. Introduction

Alzheimer's disease (AD) is a progressive, chronic, neurodegenerative disorder that is characterized by a loss of memory and cognition, severe behavioral abnormalities and ultimately death. It is the fourth leading cause of death in Western countries. As general health care improves, and the proportion of the elderly increases, the number of AD patients is anticipated to increase dramatically [1]. The neuropathological hallmarks of the disease is the presence of numerous senile amyloid β-peptide (Aβ) plaques, neurofibrillary tangles (NFT), the dramatic loss of synapses and degeneration or atrophy of the basal forebrain cholinergic neurons [2]. The loss of basal forebrain cholinergic cells results in sharp reduction in acetylcholine (ACh), which is believed to play an important role in the cognitive impairment associated with AD [3–5]. On this basis, the cholinergic hypothesis has become the leading strategy for the development of anti-AD agents. Several anti-AChE agents such as tacrine, donepezil, propidium and ensaculin (Fig. 1) have shown to induce a modest improvement in memory and cognitive functions [6]. However, these compounds do not appear to prevent or slow down the progressive neurodegeneration. The optimal approach is also being followed in searching new agents which can be used to treat AD [7].

Recent study showed that AChE could also play a key role in accelerating Aβ plaques deposition [8,9]. It is likely that AChE interacts with Aβ and promotes amyloid fibril forma-

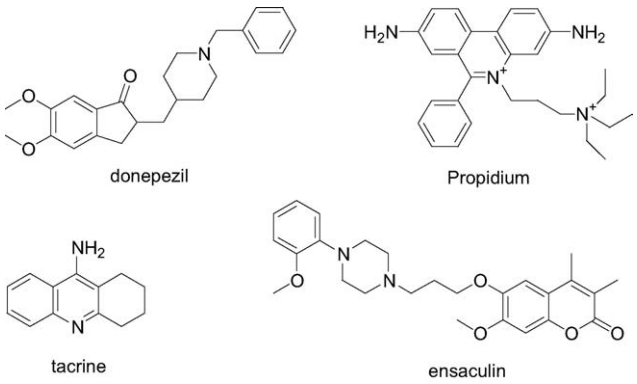

Fig. 1. Structures of compounds which have shown anti-AChE activities.

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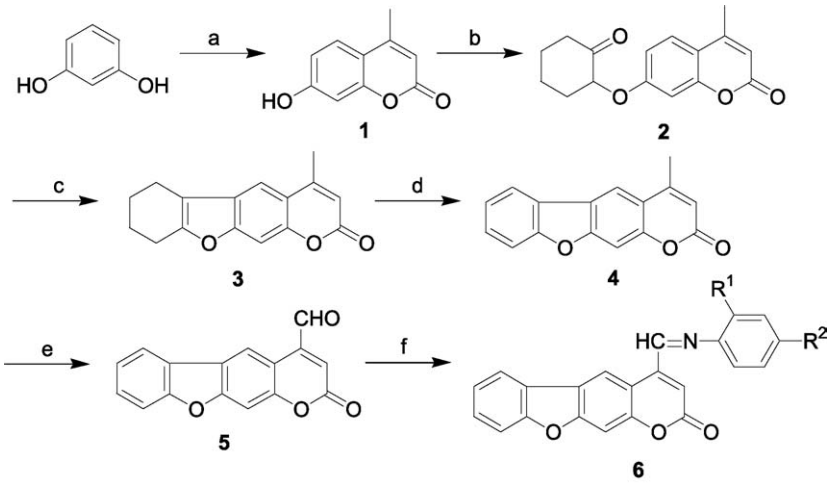


Scheme 1. Preparation of **6a**–**6n**.

tion through a pool of amino acids located in proximity of the peripheral anionic binding site (PAS) of the enzyme [10]. Moreover, it has been shown that molecules are able to interact either exclusively with PAS or with both catalytic and peripheral binding site, which can prevent the pro-aggregation of AChE toward Aβ [8]. Compounds showing peripheral and dual binding with AChE are intriguing and could represent a new type of therapeutic agents through the prevention of Aβ aggregation.

In view of the above reasons, we focused on the development of more active and selective compounds which are able both to reduce the cognitive deficiency and to decrease Aβ production or aggregation. To this aim, three series of new inhibitors were designed and prepared, their anti-AChE and BuChE activities were tested and the SARs were discussed. The results of the in vitro assays compared favorably with the corresponding data of propidium and tacrine, indicating that **6b** and **7b** might be regarded as the prototype of a new class of compounds endowed with AChE inhibitory profile that may favor for the therapeutic application in the treatment of AD.

2. Inhibitor design

A set of molecules composed of three moieties optimal for the binding at PAS, catalytic site and linked by an appropriate distance were designed to obtain selective nonhepatotoxic AChE inhibitor. The three moieties were the benzylamino or arylamino group, benzofuran group and coumarin (2*H*-2-Chromenone) skeleton. Coumarins were selected as the parent nucleus for several reasons. At first, coumarins are members of the set of compounds called benzopyrones which display a variety of pharmacological properties [11], and some functionalized coumarins are presented as potent AChE inhibitor [12,13]. The second reason is that certain functionalized coumarin derivatives are monoamine oxidase B (MAO-B) inhibitors and have also been proposed for the treatment of AD [14,15]. The third reason is the fact that coumarin compounds have demonstrated high anti-AChE potency [12,16]. Studies also showed that benzylamine substructure were found in many potent AChE inhibitors [17].

3. Chemistry

In general, compounds of series 1 (compounds **6a**–**6n**) were synthesized via a six-step procedure as described in Scheme 1. Resorcinol and ethylacetoacetate were first treated under Pechmann conditions [18] to give substituted coumarin **l**, and compound **1** was reacted with 2-chlorocyclohexanone under Williamson conditions [19] to give the corresponding oxo ether **2** under refluxed conditions.

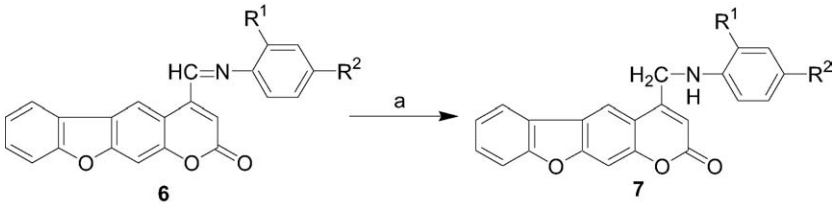
Reagents and conditions: (1) $CH_3COCH_2COOC_2H_5$, H_2SO_4, 0–5 °C; (b) dry K_2CO_3, dry acetone, KI,  reflux; (c) NaOH, reflux, 2 h; (d) DDQ, Pd/C, toluene, reflux, 8 h; (e) SeO_2, xylene, reflux, 29 h; (f) 95% ethanol, arylamine, reflux, 2 h.

Thereafter, cyclization of oxo ether **2** by heating in strong alkaline solution afforded compound **3.** This compound was aromatized in toluene with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and a catalytic amount of Pd/C to give the corresponding compound **4**. Selective oxidation of compound **4** to 4-carbaldehyde **5** was achieved using selenium dioxide as oxidant in refluxing xylene. Finally, then reaction of **5** with arylamine in 95% ethanol gave target compounds **6a–n** as pure *E*-isomers [20].

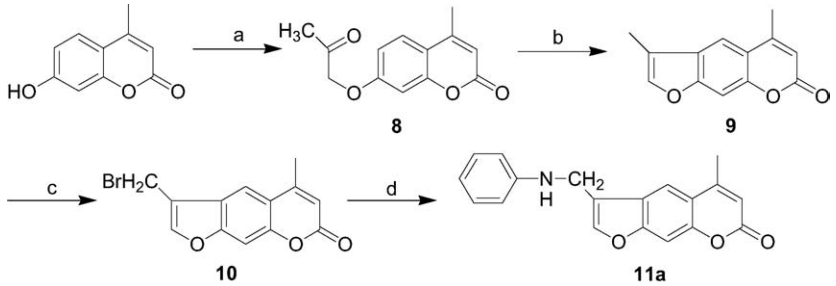
Compounds **6** were reduced with $NaBH_4$, producing compounds of series B (**7a**–**c**) (Scheme 2).

Reagents and conditions: (a) THF, methanol, $NaBH_4$, 0–10 °C.

To further verify the above hypothesis, another new target compound **11a** was also prepared according Scheme 3. The compound **11a** was obtained using the method similar to that as shown in Scheme 1. Intermediate **9** was treated with 1,3-dibromo-5,5-dimethylhydantoin (BDH) and a catalytic amount of benzoyl peroxide in refluxing CCl_4, bromination



Scheme 2. Preparation of compounds **7**.



Scheme 3. Preparation of compound **11a**.

of the methyl substituent on the furan ring gave 6-bromomethylfuro[3,2-g] coumarin (**10**). Bromination of 4,6-dimethylfuro[3,2-g] coumarin (compound **9**) occurs only in the *6*-methyl group, and the *4*-methyl group is intact during the reaction [21]. Then the bromo-derivative **10** was condensed with aniline to afford the target compound **11a**.

Reagents and conditions: (a) dry K_2CO_3, KI, CH_3COCH_2Cl, reflux, 18 h; (b) NaOH, reflux, 2 h; (c) BDH, $(PhCOO)_2$, CCl_4, 6 h; (d) aniline, toluene, CH_3CN, powdered KOH, reflux.

4. Biology

The inhibitory activities of the thus prepared compounds against AChE and BChE were studied for determining the rate of hydrolysis of acetylthiochoine (ATCh) and butylthiocholine (BuTCh) in comparison with reference compound tacrine and propidium using the method of Ellman et al. [22]. The results were listed in Table 1 as IC_{50} values. The nature of AChE inhibition caused by these compounds was investigated by the graphical analysis of steady-state inhibition data (only **6b** and **7b** were shown in Fig. 1), and the K_i values and the type of inhibition of the selected compounds were reported in Table 2 along with the data for tacrine and propidium. Structural features of the three types of compounds are shown in Fig. 2.

Table 1
AChE and BuChE inhibitory Activities (IC_{50}, μM) of prepared compounds

Compound	K_i ± S.E. (nM)	Type of inhibition
6b	0.20 ± 0.02	Non-competitive
7b	0.061 ± 0.003	Mixed
Propidium	8.13 ± 1.50	Non-competitive
Tacrine	0.15 ± 0.016	Mixed

[a] Not determined.

5. Discussion

According to the screening data, the analogues of compound **6** which possessed strong electron-donating groups, such as $–OCH_3$ in positions 2′ and 4′, $–NH_2$ in position 2′, –OH in position 2′, exhibited anti-AChE activity, while compound **6b** with a strongest electron-donor group $–OCH_3$ in the 2′-position exhibited the highest activity against AChE. Analogues of compounds **6** with weaker electron-donating group $-CH_3$ in position 2′ and 4′ showed a decreasing inhibitory activity, which suggests that strong electron-donating group in these compounds could help them to access to the enzyme-binding site. Possess two substituent groups in 2′ and 4′ positions exhibited a decreasing in AChE inhibitory effect compared to those bearing either one group in either 2′- or 4′-positions. The congeners with bulkier substituent (compound **6j**) led to a huge decrease in activity than that bearing small ones (compound **6i**), indicating the steric hindrance could limit the access of compounds to the enzyme-binding site. These phenomena implied that both the steric and electronic effects might play a significant role in influencing the activity.

Compounds **7a**, **7b** and **7c** showed significantly increased inhibitory activity compared with their unsaturated counterparts **6a**, **6b** and **6c**. These results suggested that the thus compounds may have strong interaction with AChE contributing from their better binding affinity to AChE. Interesting that the compounds **7a**, **7b** and **7c** were less potent in inhibitory activity against BuChE comparing with AchE, indicating that these compounds can serve as selective inhibition agents for AChE over BuChE. Comparing the selectivity of **7a**, **7b** and **7c** for AChE/BuChE, it is clear that the 2-methoxy group on the aromatic ring is endowed with optimal electronic properties, that agreement with above SARs results.

Compound **11a** showed less activity than compound **6b** and **7b** as an AChE inhibitor, suggested that the C-type com-

Table 2
Inhibition constant and type of inhibition of selected compounds[a]

Compound	Structural Feature	R^1	R^2	IC_{50} ± SEAChE (μM)	IC_{50} ± SEBuChE (μM)	Selectivity for AChE/BChE
6a	A	H	H	1.08 ± 0.1	nd[a]	
6b		OCH_3	H	0.43 ± 0.02	< 20	
6c		H	OCH_3	0.75 ± 0.02	< 20	
6d		CH_3	H	1.30 ± 0.04	nd	
6e		H	CH_3	1.87 ± 0.1	nd	
6f		CH_3	CH_3	2.53 ± 0.1	nd	
6g		OH	H	0.67 ± 0.02	< 20	
6h		H	OH	3.01 ± 0.2	nd	
6i		OH	CH_3	2.01 ± 0.1	nd	
6j		OH	$C(CH_3)_3$	3.21 ± 0.2	nd	
6k		H	SH	1.88 ± 0.1	nd	
6l		COOH	H	4.85 ± 0.2	nd	
6m		H	COOH	7.47 ± 0.3	nd	
6n		NH_2	H	0.88 ± 0.02	< 20	
7a	B	H	H	0.49 ± 0.02	58.6 ± 4.5	119.60
7b		OCH_3	H	0.19 ± 0.01	185 ± 13.2	973.68
7c		H	OCH_3	0.21 ± 0.01	37.8 ± 3.3	180.0
11a	C	H	H	3.02 ± 0.2	25.30 ± 2.2	8.38
Propidium				15.30 ± 0.3	nd	
Tacrine				0.29 ± 0.03	0.0568 ± 0.2	0.20

[a] Inhibition constants were calculated from kinetic data.

pound have weaker interaction with AChE, due to their poorer binding affinity to AChE.

The kinetic studies showed that the selected compound **7b** display the mixed-types of inhibition similar to that of tacrine [23], and the compound **6b** showed as non-competitive binding mode that similar to that of propidium [23,24].

Compounds **7a**, **7b** and **7c** showed significantly increased activity compared with their unsaturated counterparts **6a**, **6b** and **6c**. These results indicated that the B-type compounds have strong interaction with AChE that may due to better binding affinity to AChE. Meanwhile, compounds **7a**, **7b** and **7c** were less potent of inhibitory activity against BuChE than that of AchE, indicating that the B-type compounds exhibit a good selectivity for AChE over BuChE.

Compound **11a** showed slightly (two to threefold) less activity than compound **6a** as an AChE inhibitor, indicating that the C-type compound have weaker binding affinity to AchE comparing with type- A or B compounds.

Reciprocal plots of initial velocity and substrate concentration are reported. Lines were derived from a weighted least-squares analysis of data points. The plots show non-competitive inhibition for **6b**, mixed-type inhibition for **7b**.

The kinetic analysis showed that compounds**7a**, **7b**, **7c** and **11a**, display the mixed-types of inhibition similar to that of tacrine [23], while the other compounds showed the binding mode of non-competitive similar to that of propidium [23,24].

The results from SARs analysis, biological assay data and kinetic studies that provided coherent information concerning the inhibitory action on AChE or BuChE and the selectivity for AChE/BuChE, those to be favor for design a new type of the prototypes.

Interestingly, Inestrosa [8] and Bartolini [23] recently pointed out a direct link between AChE and Aβ aggregation. AChE was shown to exert an Aβ pro-aggregation mediated by its peripheral binding site [10]. We found that compound **6b** with the binding mode similar to that of propidium and showed the potency inhibitory activity against AChE [25]. This finding is particular interesting in the connection to the Alzheimer's disease, because recent observations show that peripheral AChE inhibitors might decrease β-amyloid deposition [8,23].

In addition, compound **7b** showed a strong potency inhibitory activity against AChE which was comparable to that of tacrine [8,23,25], and also demonstrated a high selectivity for AChE over BChE. This remarkable selectivity features may have some implication on the future design and preparation of AChE inhibitors [23]. Actually, a peculiar structural difference between the two types of cholinesterases is the lack

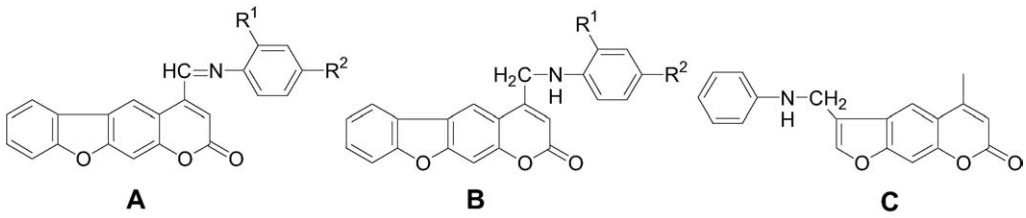


Fig. 2. Structural features of three types of compounds.

of the PAS (in particular of Trp286) in BuChE [26], that seems to prevent the interaction of BuChE with Aβ, resulting ineffective on Aβ aggregation [8]. For instance, tacrine, a non-competitive mixed-type AChE inhibitor, binds more tightly to BuChE and is almost inactive in inhibiting the AChE-promoted Aβ aggregation [23,26]. This inhibitor mostly interacted with AChE catalytic site [27], as it likely did with BuChE [26]. In contrast, propidiun, a well-known AChE inhibitor which binding exclusively at the PAS, was almost inactive against BuChE and strongly inhibits the AChE-induced Aβ aggregation [23]. The results presented in Tables 1 and 2 suggested the phenomenological correlation between AChE selectivity and inhibition of AChE-induced Aβ aggregating action. A inhibitors of AChE that to be strongly interacting with the PAS, that would be shown high AChE/BuChE selectivity, and eventually performed inhibitory effect against AChE-induced Aβ aggregation Fig. 3.

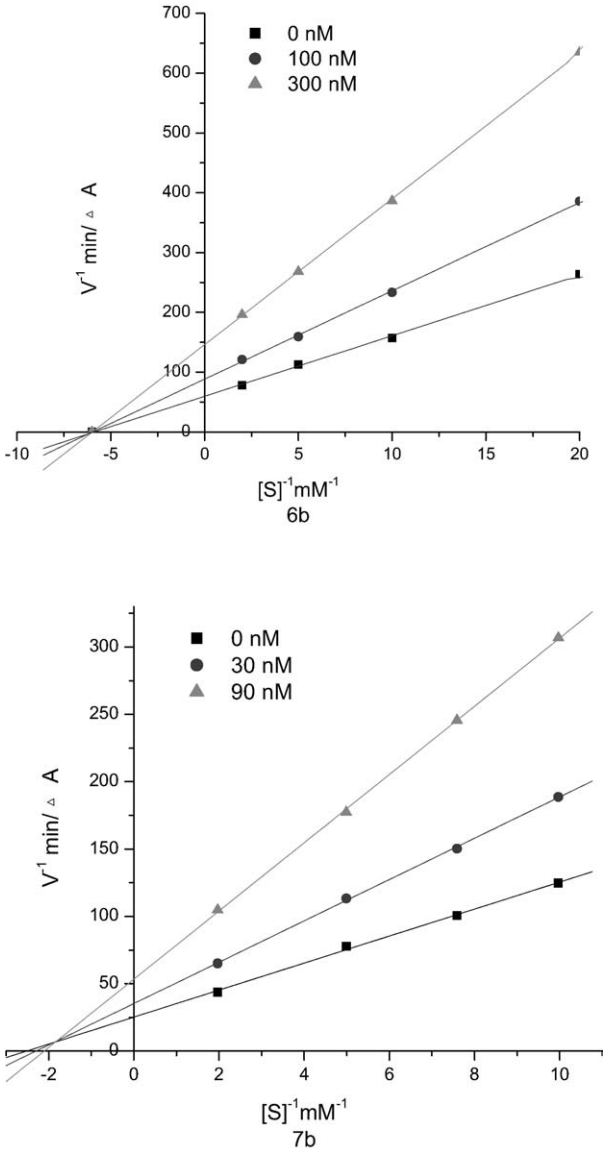


Fig. 3. Steady state inhibition of **6b** and **7b** against AchE.

6. Conclusion

In conclusion, we have prepared three series of compounds and studied the enzyme-inhibitor binding mode and the structure–activity relationship. We hope that the current work could shed light on further investigations on the potential AChE inhibitors with high inhibitory activity and good selectivity for AchE over BuChE.

7. Experimental section

7.1. Chemistry

Melting points were measured using an electro-thermal 8103 apparatus and were uncorrected. IR spectra were taken with Perkin-Elmer 398 spectrometers. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Unity INOVA 500 MHz spectrometer with TMS as internal standard. All chemicals used were of analytical grade. TLC monitored the progress of reactions using pre-coated 60 F_{254} silica gel plates (0.25 mm, Merck). Elemental analyses (C, H, N) were performed on a Perkin-Elmer 240 elemental analyzer, and the results were within ± 0.4% of the theoretical values. ESI-MS spectra were performed on a Thermo Finigan LCQ DECA XP ion trap mass spectrometer.

7.2. Synthesis of A-type compounds (*6a–n*)

4-Methyl-7-hydroxycoumarin (**1**). Compound **1** was prepared based on a literature method [18]. Yield 80%, m.p. 190–192 °C; IR(KBr) $\tilde{\nu}$: 3159, 1679, 1652, 1600, 1451 cm^{-1}; FAB-MS *m/z*:177([M + H]$^{+}$), 154([M–OH]$^{+}$); ^{1}H NMR(CDCl$_3$)δ: 9.31 (s, 1H, 7-OH), 7.61 (d, 1H, J = 8.5, 1.0 Hz, 5-H), 6.85 (m, 1H, 6-H), 6.74 (d, 1H, J = 2.5 Hz, 8-H), 6.07 (s, 1H, 3-H), 2.42 (s, 3H, 4-CH$_3$), ^{13}C NMR (CDCl$_3$) δ: 161.8 (C$_2$), 160.9 (C$_7$), 156.3 (C$_{8a}$), 153.6 (C$_4$), 113.4 (C$_{4a}$), 111.8 (C$_5$), 106.4 (C$_6$), 103.3 (C$_8$), 96.6 (C$_3$), 18.5 (CH$_3$).

4-Methyl-7-(2′-oxocyclohexyloxy)coumarin (**2**). Compound **2** was prepared based on a literature method [19]. Yield 80%, m.p. 164–166 °C; IR(KBr) $\tilde{\nu}$: 3050, 2922, 2853, 1716, 1605, 1574, 1432, 747, 717 cm^{-1}; FAB-MS *m/z*: 273 ([M + H]$^{+}$), 154([M-C$_6$H$_7$H$_2$O]$^{+}$); ^{1}H NMR (DMSO-d_6) δ: 7.62 (d, 1H, J = 8.5 Hz, 5-H), 6.88 (dd, 1H, J = 6.0, 2.5 Hz, 6-H), 6.75 (s, 1H, 8-H), 5.13 (m, 1H, 1′-H), 2.76–2.65 (m, 2H, 3′-H), 2.48–2.43 (m, 2H, 6′-H), 2.42–2.38 (m, 3H, 4-CH$_3$), 2.12–1.89 (m, 4H, 4’-H, 5’-H); ^{13}C NMR (CD$_3$OD) δ: 200.8 (C$_{2'}$), 162.2 (C$_2$), 160.8 (C$_7$), 156.1 (C$_{8a}$), 153.6 (C$_4$), 126.8 (C$_5$), 114.4 (C$_{4a}$), 113.6 (C$_6$), 112.4 (C$_8$), 102.7 (C$_3$), 81.1 (C$_{1'}$), 41.3 (C$_{3'}$), 35.0 (C$_{4'}$), 28.1 (C$_{5'}$), 24.1 (C$_{6'}$), 18.5 (CH$_3$).

6,7,8,9-Tetrahydro-4-methyl-*2H*-benzofuro [3,2-g]-1-benzopyran-2-one (**3**). A solution of **2** (10.0 mmol) in 0.8 M NaOH (1L) was refluxed in the dark for 3 h under N$_2$. The solution was cooled, diluted with water (1 l), and acidified with diluted HCl. The precipitate obtained was collected and crystallized from MeOH to give **3**. Yield 95%, m.p. 190–

192 °C; IR (KBr) $\tilde{\nu}$: 3053, 2934, 2886, 1711, 1628, 1573, 1458, 1292, 875, 804 cm^{-1}; FAB-MS *m/z*: 255 ([M + H]$^+$), 154([M-$C_6H_9H_2O$]$^+$); ^{1}H NMR ($CDCl_3$) δ: 7.46 (s, 1H, 5-H), 7.28 (s, 1H, 11-H), 6.17(s, 1H, 3-H), 2.75–2.72 (m, 2H, 9-H), 2.63–2.61(m, 2H, 6-H), 2.45 (s, 3H, 4-CH_3), 1.98–1.85 (m, 4H, 7-H, 8-H); ^{13}C NMR ($CDCl_3$) δ: 161.2 (C_2), 156.2 (C_{10a}), 155.8 (C_{9a}), 152.8 (C_{11a}), 150.9 (C_{4a}), 126.2 (C_{5b}), 115.6 (C_{5a}), 113.1 (C_5), 112.6 (C_{11}), 99.2 (C_3), 23.3 (C_6), 22.6 (C_8), 22.3 (C_7), 20.2 (C_9), 19.1 (CH_3).

4-methyl-*2H*-benzofuro[3,2-g]-1-benzopyran-2-one (**4**). A mixture of **3** (10 mmol), 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (25 mmol) and a catalytic amount of Pd/C in anhydrous toluene (500 ml) was refluxed for 6 h. After cooling, the solid was filtered off and the solvent evaporated under reduced pressure. The residue was purified by column chromatography and crystallized from MeOH to give **4**. Yield 98%, m.p. 228–230 °C; IR(KBr) $\tilde{\nu}$: 3099, 2940, 2870, 1715, 1617, 1553, 1513, 1437, 1294, 1265, 1206, 1148, 1082, 842 cm^{-1}; FAB-MS *m/z*: 251 ([M + H]+),154 ([M-$C_6H_92H_2O$]+); ^{1}H NMR ($CDCl_3$) δ: 8.11 (s, 1H, 5-H), 7.98 (dd, 1H, *J* = 7.0, 1.0 Hz, 6-H), 7.58 (d, 1H, *J* = 8.0 Hz, 9-H), 7.51 (t, 1H, *J* = 7.5 Hz, 7-H), 7.50 (s, 1H, 11-H), 7.40 (t, 1H, *J* = 15.0 Hz, 8-H), 6.29 (s, 1H, 3-H), 2.57 (s, 3H, 4-CH_3); ^{13}C NMR ($CDCl_3$) δ: 160.8 (C_2), 157.9 (C_{10a}), 157.2 (C_{9a}), 153.4 (C_{11a}), 152.5 (C_4), 127.8 (C_6), 123.5 (C_{5b}), 123.2 (C_8), 121.6 (C_{4a}), 120.6 (C_7), 116.2 (C_{5a}), 116.0 (C_3), 113.5 (C_5), 111.9 (C_9), 100.2 (C_{11}), 19.2 (CH_3).

4-Carbaldhyde-*2H*-benzofuro [3,2-g]-1-benzopyran-2-one (**5**). Powdered SeO_2 (3.30 g, 30 mmol) was added to a solution of **4** (20 mmol) in 20 ml of hot dry xylene and the mixture were refluxed for 24 h with vigorous stirring. The reaction mixture was filtered to remove black Se, and the deep orange filtrate was allowed to stand overnight. Almost pure crystals of **5** could be separated from the solution. Yield 90%, m.p. 259–261 °C; IR(KBr)$\tilde{\nu}$: 3056, 2854, 2717, 1730, 1639, 1600, 1586, 1453, 1433, 1402, 1380, 1339, 1302, 1267, 1212, 1048, 850, 800 cm^{-1}; FAB-MS *m/z*: 265 ([M + H]$^+$), 168 ([M-C_6H_8O]$^+$); ^{1}H NMR($CDCl_3$) δ: 10.17 (s, 1H, CHO), 9.22 (s, 1H, 5-H), 8.04 (d, 1H, *J* = 7.5 Hz, 6-H), 7.61–7.22 (m, 4H, 7-H, 8-H, 9-H, 11-H), 6.88 (s, 1H, 3-H); ^{13}C NMR ($CDCl_3$) δ: 191.9 (CHO), 160.3 (C_2), 158.2 ($C1_{0a}$), 157.3 (C_{9a}), 154.3 (C_{11a}), 143.9 (C_4), 128.2 (C_3), 124.3 (C_{5b}), 123.8 (C_{4a}), 123.1 (C_{5a}), 122.7 (C_6), 121.1 (C_8), 118.4 (C_7), 111.9 (C_5), 110.6 (C_9), 100.5 (C_{11}).

General procedure for preparation of compounds **6**. A mixture of **5** (5 mmol) and arylamine (15 mmol) in 95% ethanol (25 ml) was refluxed for 2 h. After cooling, the precipitate was collected, washed with 95% ethanol, and recrystallized to give **6** in the yield of 35–90%.

4-((Phenylimino)methyl)-2*H*-benzofuro[3,2-g]chromen-2-one(**6a**). m.p. 238–240 °C; FAB-MS *m/z*: 340 ([M + H]$^+$); ^{1}H NMR(THF-d_8) δ: 9.57 (s, 1H, HC=N), 8.88(s, 1H, 5-H), 8.05(d, 1H, *J* = 9.5 Hz, 9-H), 7.58 (m, 2H, 6-H, 11-H), 7.50–7.42 (m, 5H, 2′-H, 3′-H, 4′-H, 5′-H, 6′-H), 7.40–7.32 (m, 2H, 7-H,8-H), 6.85 (s, 1H, 3-H). C, H, N for $C_{22}H_{13}NO_3$:

4-((2-Methoxyphenylimino)methyl)-2*H*-benzofuro[3,2-g]chromen-2-one(**6b**). m.p. 172–174 °C; IR(KBr)$\tilde{\nu}$: 3331, 3067, 3001, 2955, 2906, 2832, 1718, 1643,1602, 1569, 1514, 1431, 1247, 1222, 1138, 781, 745 cm^{-1}; FAB-MS *m/z*: 370 ([M + H]$^+$), 171 ([M-C6H4.C_7H_7O]$^+$); ^{1}H NMR (THF-d_8) δ: 9.77 (s, 1H, HC=N), 8.83 (s, 1H, 5-H), 8.02(d, 1H, *J* = 7.0 Hz, 9-H), 7.58(d, 1H, *J* = 8.5 Hz, 6-H), 7.55 (s, 1H, 11-H), 7.47 (t, 1H, *J* = 15.0 Hz, 7-H), 7.37(t, 1H, *J* = 15.0 Hz, 8-H), 7.28–7.11 (m, 2H, 4′-H, 6′-H), 7.00 –6.86 (m, 2H, 3′H, 5′-H), 6.76 (s, 1H, 3-H), 3.97 (s, 3H, OCH_3); ^{13}C NMR (THF-d_8) δ: 160.2 (C_2), 159.0 (HC=N), 158.6 (C_{10a}), 158.2 ($C_{2'}$), 155.6 (C_{9a}), 154.1 (C_{11a}), 146.6 ($C_{1'}$), 141.1 (C_{5b}), 129.2 (C_3), 128.5 ($C_{4'}$), 124.3 (C_6), 122.2 (C_{4a}), 121.7 (C_6), 121.5 (C_7), 121.2 (C_5), 120.5 (C_9), 119.2 ($C_{6'}$), 117.9 (C_{5a}), 117.4 (C_4), 113.0 ($C_{3'}$), 112.4 ($C_{5'}$), 100.5 (C_{11}), 56.3(OCH_3). C, H, N for $C_{23}H_{15}NO_4$.

4-((4-Methoxyphenylimino)methyl)-2H-benzofuro[3,2-g]chromen-2-one(**6c**). m.p. 244–245 °C; IR(KBr) $\tilde{\nu}$: 3064, 2951, 2842, 1705, 1643, 1604, 1554, 1503, 1436, 1250, 1197, 1133, 875 cm^{-1}; FAB-MS *m/z*: 370 ([M + H]$^+$),154 ([M-$C_6H_4OC_7H_7NO$]$^+$); ^{1}H NMR (THF-d_8) δ: 9.46 (s, 1H, HC=N), 8.93 (s, 1H, 5-H), 8.08 (d, 1H, *J* = 8.5 Hz, 6-H), 7.60 (s, 1H, 11-H), 7.51–7.47 (m, 3H, 8-H, 2′-H, 6′-H), 7.38(t, 1H, *J* = 7.5 Hz, 7-H), 7.03 (dd, 2H, *J* = 6.5, 7.0 Hz, 3′-H, 5′-H), 6.83 (s, 1H, 3-H), 3.84 (s, 3H, OCH_3); ^{13}C NMR (THF-d_8) δ: 161.2 (C_2), 160.3 (C_{10a}), 158.8 (C_{9a}), 158.3 (C_{11a}), 155.8 ($C_{4'}$), 155.5 (HC=N), 146.7 ($C_{1'}$), 144.5 (C_{5b}), 128.5 (C_3), 124.6 (C_{4a}), 124.3 (C_6), 123.9 ($C_{2'}$,$C_{6'}$), 122.3 (C_4), 121.7 (C_8), 119.8 (C_7), 118.3 (C_5), 115.4 ($C_{3'}$,$C_{5'}$), 114.4 (C_{5a}), 112.5 (C_9), 100.7 (C_{11}). C, H, N for $C_{23}H_{15}NO_4$:

4-((*o*-Tolylimino)methyl)-2*H*-benzofuro[3,2-g]chromen-2-one (**6d**). m.p. 260–262 °C; IR(KBr) $\tilde{\nu}$: 3059, 3025, 2914, 1703, 1644, 1604, 1560, 1504, 1433, 1266, 1200, 1137, 742 cm^{-1}; FAB-MS *m/z*: 354([M + H]$^+$), 154([M-$C_6H_4O.C_7H_7N$]$^+$); ^{1}H NMR ($CDCl_3$) δ: 9.66 (s, 1H, HC=N), 9.08 (s, 1H, 5-H), 7.68 (s, 1H, 11-H), 7.65 (d, 1H, *J* = 9.5 Hz, 9-H), 7.56–7.49 (m, 2H, 7-H, 8-H), 7.49–7.39 (m, 2H, 4′-H, 6′-H), 7.31(m, 2H, 3′-H, 5′-H), 6.97 (s, 1H, 3-H), 2.52–2.51(m, 3H, CH_3); ^{13}C NMR (THF-d_8) δ: 160.8 (C_2), 158.1 (C_{10a}), 157.3 (HC=N), 155.5 (C_{9a}), 151.0 (C_{11a}), 149.0 ($C_{1'}$), 147.0 ($C_{2'}$), 138.9(C_{5b}), 130.8 ($C_{4'}$), 130.0 (C_{4a}), 128.9 (C3), 124.6 ($C_{5'}$), 124.4 (C_{5a}), 122.7 (C_6), 122.4 (C_4), 122.0 (C_8), 120.0 ($C_{3'}$), 119.9 ($C_{6'}$), 117.9 (C_7), 112.6 (C_5), 100.8 (C_9), 88.3 (C_{11}), 21.3 (CH_3). C, H, N for $C_{23}H_{15}NO_3$:

4-((*p*-Tolylimino)methyl)-2*H*-benzofuro[3,2-g]chromen-2-one (**6e**). m.p. 226–228 °C; IR (KBr) $\tilde{\nu}$:3055, 1706, 1642, 1604, 1564, 1486, 1267, 1138, 884, 845 cm^{-1}. FAB-MS *m/z*: 354([M + H]$^+$),154([M-$C_6H_4O.C_7H_7N$]$^+$); ^{1}H NMR ($CDCl_3$) δ: 9.70 (s, 1H, HC=N), 8.98 (s, 1H, 5-H), 8.05 (d, 1H, *J* = 8.0 Hz, 9-H), 7.84 (s, 1H, 11-H), 7.74 (d, 1H, *J* = 8.0 Hz, 9-H), 7.57 (t, 1H, *J* = 15.0 Hz, 8-H), 7.46 (t, 1H, *J* = 15.0 Hz, 7-H), 7.38–7.27 (m, 4H, 2′-H, 5′-H, 6′-H), 7.02 (s, 1H, 3-H), 2.50 (s, 3H, CH_3); ^{13}C NMR (CH_3OD) δ: 166.5(C_2), 165.9 (C_{10a}), 163.2 (C_{9a}), 158.4(C_{11a}), 155.2 ($C_{1'}$), 142.2 (HC=N), 139.9 ($C_{4'}$), 137.5 (C_{1a}), 136.9(C_3), 136.3 (C_{5b}), 133.3 (C_{4a}), 132.2 (C_5a), 130.4 ($C_{3'}$, $C_{5'}$), 128.4 (C_6), 127.0 ($C_{2'}$, $C_{5'}$), 126.3 (C_8), 122.5 (C_7), 121.2 (C_5), 109.6 (C_9), 104.8 (C_{11}), 27.3(CH_3). C, H, N for $C_{23}H_{15}NO_3$.

4-((2,4-Dimethylphenylimino)methyl)-2*H*-benzofuro[3,2-g]chromen-2-one (**6f**). m.p. 247–249 °C; ^{1}H NMR (THF-d_8)

δ: 9.78 (s, 1H, CH = N), 8.84 (s, 1H, 5-H), 8.04 (d, 1H, J = 7.2 Hz, 9-H), 7.64 (s, 1H, 6′-H), 7.62 (d, 1H, J = 5.0 Hz, 6-H), 7.50–7.05 (m, 5H, 7-H, 8-H, 11-H, 3′-H, 5′-H), 6.84 (s, 1H, 3-H), 2.53 (s, 3H, 2′-CH_3), 2.40 (s, 3H, 4′-CH_3). C, H and N for $C_{24}H_{17}NO_3$.

(2-Hydroxyphenylimino)methyl)-2*H*-benzofuro[3,2-g]chromen-2-one (**6g**). m.p. 258–259 °C; IR (KBr) $\tilde{\nu}$: 3446, 3064, 1711, 1644, 1593, 1550, 1483, 1454, 1249, 1200, 1139, 1105, 799, 744.5 cm^{-1}; FAB-MS *m/z*: 356([M + H]$^+$), 171 ([M-C_6H_4.C_6H_5O]$^+$); ^{1}H NMR (THF-d_8) δ: 9.41 (s, 1H, HC=N), 9.20 (s, 1H, OH), 8.42 (s, 1H, 5-H), 8.09 (dd, 1H, J = 7.5, 2 Hz, 6-H), 7.63 (s, 1H, 11-H), 7.62 (d, 1H, J = 3.5 Hz, 9-H), 7.51 (t, 1H, J = 7.5 Hz, 7-H), 7.47 (t, 1H, J = 12.0 Hz, 8-H), 7.39 (t, 1H, J = 10.0 Hz, 6′-H), 7.22 (t, 1H, J = 15.0 Hz, 3′-H), 7.06 (s, 1H, 3-H), 6.97 (d, 1H, J = 8.0 Hz, 5′-H), 6.91 (t, 1H, J = 15.0 Hz, 4′-H); ^{13}C NMR (THF-d_8) δ: 160.3 (C_2), 158.8 (C_{10a}), 158.2 (C_{9a}), 155.6 (C_{11a}), 154.2 (HC=N), 146.7 ($C_{2'}$), 137.6 ($C_{1'}$), 130.7 (C_3), 128.6 ($C_{4'}$), 124.5 (C_{5b}), 124.4 (C_6), 122.3 (C_{4a}), 121.7 (C_8), 120.5 (C_4), 119.2 (C_5), 119.1 (C_9), 117.4 ($C_{6'}$), 116.7 ($C_{5'}$), 114.6 (C_{5a}), 112.5 ($C_{3'}$), 100.7 (C_{11}). C, H, N for $C_{22}H_{13}NO_4$.

4-((4-Hydroxyphenylimino)methyl)-2*H*-benzofuro[3,2-g]chromen-2-one (**6h**). m.p. 254–255 °C; IR (KBr) $\tilde{\nu}$: 3601, 3380, 3343, 3094, 3028, 1692, 1642, 1602, 1574, 1506, 1475, 1456, 1387, 1341, 1266, 1218, 1139, 1099, 839, 810 cm^{-1}; FAB-MS *m/z*: 356 ([M + H]$^+$),154 ([M-C_6H_4O.C_6H_6NO]$^+$); ^{1}H NMR (THF-d_8) δ: 9.64 (s, 1H, HC=N), 8.88 (s, 1H, 5-H), 8.50(s, 1H, OH), 8.07–8.05(m, 1H, 6-H), 7.84 (s, 1H, 11-H), 7.62–7.48 (m, 2H, 8-H, 7-H), 7.47–7.40 (m, 2H, 2′-H, 6′-H), 6.89–6.88 (m, 2H, 3′-H, 5′-H), 6.80(s, 1H, 3-H); ^{13}C NMR (THF-d_8) δ: 160.3 (C_2), 159.4 (C_{10a}), 158.7 (C_{9a}), 158.2 ($C_{4'}$), 155.7 (C_{11a}), 154.3 (HC=N), 150.3 ($C_{1'}$), 143.3 (C_{5b}), 128.5 (C_3), 124.6 (C_4), 124.2 ($C_{2'}$, $C_{6'}$), 122.2 (C_{4a}), 121.6 (C_6), 119.8 (C_8), 117.9(C_7), 116.8 ($C_{3'}$, $C_{5'}$), 116.2 (C_5), 114.5 (C_{5a}), 112.5 (C_9), 100.6 (C_{11}). C, H, N for $C_{22}H_{13}NO_4$.

4-((2-Hydroxy-4-methylphenylimino)methyl)-2*H*-benzofuro[3,2-g]chromen-2-one (**6i**). m.p. 208–210 °C; FAB-MS *m/z*: 370 ([M + H]$^+$). ^{1}H NMR (THF-d_8) δ: 10.05 (s, 1H, OH), 9.27 (s, 1H, HC=N), 9.02 (s, 1H, 5-H), 7.96 (d, 1H, J = 7.8 Hz, 9-H), 7.94 (d, 1H, J = 7.5 Hz, 6-H), 7.53 (t, 2H, J = 12.0 Hz, 4′-H, 6′-H), 7.40 (t, 1H, J = 6.9 Hz, 7-H), 7.29 (t, 1H, J = 6.9 Hz, 8-H), 7.14 (t, 1H, J = 6.9 Hz, 7-H), 6.94 (t, 1H, J = 2.9 Hz, 3′-H), 6.75(s, 1H, 3-H), 3.97(s, 1H, 3-H). C, H, N for $C_{23}H_{15}NO_4$.

4-((4-*tert*-Butyl-2-hydroxyphenylimino)methyl)-2H-benzofuro[3,2-g]chromen-2-one (**6j**). m.p. 243–245 °C; ^{1}H NMR (THF-d_8) δ: 10.56 (s, 1H, OH), 9.42 (s, 1H, CH = N), 9.02 (s, 1H, 5-H), 8.06 (t, 2H, J = 1.2 Hz, 6-H, 9-H), 7.68 (s, 1H, 11-H), 7.57–7.21 (m, 4H, 7-H, 8-H, 6′-H, 5′-H), 6.82 (s, 1H, 3-H), 3.61 (s, 9H). C, H, N for $C_{26}H_{21}NO_4$.

4-((4-Mercaptophenylimino) methyl)-2H-benzofuro[3,2-g] chromen-2-one (**6k**). m.p. 191–193 °C; ^{1}H NMR (THF-d_8) δ: 10.12 (s, 1H, SH), 9.23 (s, 1H, CH = N), 8.95 (s, 1H, 5-H), 8.17–6.92 (m, 9H), 6.71 (s, 1H, 3-H). C, H, N for $C_{22}H_{13}NO_3S$.

2-((2-Oxo-2*H*-benzofuro [3,2-g] chromen-4-yl) methyl-eneamino)benzoic acid (**6l**). m.p. 256–258 °C; IR(KBr) $\tilde{\nu}$: 3473, 3307, 3077, 1716, 1643, 1611, 1574, 1488, 1437,1288, 1215, 1143, 792, 750 cm^{-1}; FAB-MS *m/z*: 384 ([M + H]$^+$), 154 ([M-$C_6H_4O.C_7H_5O_2N$]$^+$); ^{1}H NMR (Pyr-d_5) δ: 10.32 (s, 1H, COOH), 9.34 (s, 1H, HC=N), 8.97 (s, 1H, 5-H), 8.40 (d, 1H, J = 8.0 Hz, 6-H), 8.56 (s, 1H, 11-H), 8.25 (d, 1H, J = 8.0 Hz, 3′-H), 7.98 (d, 1H, J = 7.5 Hz, 9-H), 7.92 (d, 1H, J = 8.0 Hz, 6′-H), 7.66–7.57 (m, 2H, 7-H, 8-H), 7.52–7.44 (m, 2H, 4′-H, 5′-H), 7.20 (s, 1H, 3-H); ^{13}C NMR (Pyr-d_5) δ: 171.6 (COOH), 158.9 (C_2), 157.5 (C_{10a}), 154.8 (HC=N), 152.7 (C_{9a}), 150.1 (C_1), 149.1 (C_{11a}), 148.8 (C_{5b}), 144.1 (C_{4a}), 134.1 ($C_{5'}$), 132.5 ($C_{4'}$), 131.1 ($C_{2'}$), 128.5 ($C_{3'}$), 128.4 (C_{5a}), 124.2 (C_3), 124.1 (C_6), 123.9 ($C_{1'}$), 122.3 (C_4), 121.5 (C_8), 118.7 ($C_{6'}$), 117.0 (C_7), 115.5 (C_9), 112.2 (C_5), 100.6 ($C_{5'}$), 96.5 (C_{11}). C, H and N for $C_{23}H_{13}NO_5$.

4-((2-Oxo-2*H*-benzofuro [3,2-g] chromen-4-yl) methyl-eneamino)benzoic acid (**6m**). m.p. 260–262 °C; IR (KBr) $\tilde{\nu}$: 3067, 2979, 2886, 2665, 2545, 1708, 1644, 1603, 1562, 1429, 1324, 1289, 1139, 1108, 857, 817 cm^{-1}. ESI-MS *m/z*: 382 ([M-H]$^-$); ^{1}H NMR (Pyr-d_5) δ: 11.74(s, 1H, COOH), 9.64 (s, 1H, HC=N), 8.99 (s, 1H, 5-H), 8.53 (d, 1H, J = 12.0 Hz, 6-H), 8.51(s, 1H, 11-H), 8.43 (d, 2H, J = 20.0 Hz, 2′-H, 6′-H), 8.06 (d, 1H, J = 7.5 Hz, 9-H), 7.72–7.62(m, 2H, 3′-H, 5′-H), 7.58 (dd, 1H, J = 8.5, 2.0 Hz, 8-H), 7.50 (ddd, 1H, J = 1.5, 7.0, 1.0 Hz, 7-H), 7.15(s, 1H, 3-H). ^{13}C NMR (Pyr-d_5) δ: 176.2 (COOH), 165.2 (C_2), 158.4 (C_{10a}), 156.8 (HC=N), 154.7 ($C_{1'}$), 153.8 (C_{9a}), 150.9 (C_{11a}), 149.2 (C_{5b}), 148.8 (C_{4a}), 131.8 ($C_{3'}$, $C_{5'}$), 130.7 ($C_{4'}$), 129.4 (C_3), 127.7 (C_{5a}), 124.4 (C_4), 123.6 (C_6), 122.4 ($C_{2'}$, $C_{6'}$), 121.3 (C_8), 120.2 (C_7), 116.7 (C_5), 113.6 (C_9), 100.8 (C_{11}). C, H and N for $C_{23}H_{13}NO_5$.

4-((2-Aminophenylimino)methyl)-2*H*-benzofuro [3,2-g] chromen-2-one (**6n**). m.p. 276–278 °C; IR (KBr) $\tilde{\nu}$: 3270, 3066, 1674, 1643, 1605, 1564, 1492, 1428, 1391, 1366, 1274, 1251, 1230, 1104, 1029, 998, 886, 851, 762 cm^{-1}; FAB-MS *m/z*: 353 ([M-H]$^+$), 154 ([M-C_6H_7O·$C_6H_4N_2$]+); ^{1}H NMR (THF-d_8) δ: 12.36(s, 1H, CH = N), 10.01(s, 1H, 5-H), 8.17 (d, 1H, J = 8.0 Hz, 6-H), 7.92(d, 1H, J = 7.5 Hz, 9-H), 7.63(s, 1H, 11-H), 7.62–7.58(m, 2H, 3′-H, 6′-H), 7.50(t, 1H, J = 8.5, 7-H), 7.41–7.30(m, 3H, 8-H, 4′-H, 5′-H), 6.88 (s, 1H, 3-H). C, H and N for $C_{22}H_{14}N_2O_3$.

*7.3. Synthesis of B-type compounds (**7a–c**)*

A suspension of $NaBH_4$ (2.2 mmol) in 3 ml of tetrahydrofuran was added to a methanolic solution of compound **6** (10 mmol) and the reaction mixture was stirred at 0 °C for 10 h. The methanol and tetrahydrofuran were evaporated and the residue was diluted with water (20 ml), acidified with diluted HCl. The precipitate obtained was collected and crystallized to give compound **7** in the yield of 75–85%.

4-((Phenylamino)methyl)-2*H*-benzofuro[3,2-g]chromen-2-one (**7a**). m.p. 296–298 °C; FAB-MS *m/z*: 342 ([M + H]$^+$); ^{1}H NMR (THF-d_8) δ: 8.32 (s, 1H, 5-H), 7.96 (d, 1H, J = 7.8 Hz, 9-H), 7.81 (d, 1H, J = 8.2 Hz, 6-H), 7.73 (t, J = 12.5 Hz, 7-H), 7.55 (t, J = 12.5 Hz, 8-H), 7.43 (s, 1H, 11-H), 7.18 (m, 2H, 3′-H, 5′-H), 7.11 (s, 1H, 4′-H), 6.87 (m, 2H, 2′-H, 6′-H), 6.62 (s, 1H, 3-H), 4.81 (t, 1H, NH), 3.85 (d, J = 12.0 Hz, CH_2N). C, H, N for $C_{22}H_{15}NO_3$.

4-((2-Methoxyphenylamino)methyl)-2*H*-benzofuro[3,2-g]chromen-2-one (**7b**). m.p. 291–293 °C; FAB-MS *m*/*z*: 372 ($[M + H]^+$), ^{1}H NMR (THF-d_8) δ: 8.27 (s, 1H, 5-H), 8.05 (d, 1H, J = 7.2 Hz, 9-H), 7.96 (d, 1H, J = 7.6 Hz, 6-H), 7.87 (t, J = 10.5 Hz, 8-H), 7.71 (t, J = 10.5 Hz, 7-H), 7.65 (s, 1H, 11-H), 7.12–6.86 (m, 4H, 3′-H, 4′-H, 5′-H, 6′-H), 6.67 (s, 1H, 3-H), 3.86 (s, 3H, OCH_3). C, H and N for $C_{23}H_{19}NO_4$.

4-((4-Methoxyphenylamino)methyl)-2*H*-benzofuro[3,2-g]chromen-2-one (**7c**). m.p. 325–327 °C; FAB-MS *m*/*z*: 372 ($[M + H]^+$), ^{1}H NMR (THF-d_8) δ: 8.31 (s, 1H, 5-H), 8.13 (d, 1H, J = 7.8 Hz, 9-H), 7.91 (d, 1H, J = 7.6 Hz, 6-H), 7.78 (t, J = 11.5 Hz, 8-H), 7.67 (t, J = 11.5 Hz, 7-H), 7.55 (s, 1H, 11-H), 7.15–6.86 (m, 4H, 2′-H, 3′-H, 5′-H, 6′-H), 6.71 (s, 1H, 3-H), 3.76 (s, 3H, OCH_3). C, H and N for $C_{23}H_{19}NO_4$.

7.4. Synthesis of C-type compound (*11a*)

7-(2-Oxo-propoxy)-4-methyl-2*H*-chromen-2-one (**8**). A mixture of **1** (60.0 mmol), chloroacetone (120.0 mmol), and anhydrous K_2CO_3 (60.0 g) in dry acetone (1000 ml) was refluxed until the disappearance of **1** (72 h, monitored by TLC). After cooling, the solid was filtered off and washed with fresh acetone. The solvent was evaporated from the combined filtrate and washings, and the residue was crystallized form MeOH to give **8** as a white crystal. Yield 67%, m.p. 161–163 °C; ^{1}H NMR ($CDCl_3$) δ: 7.66–7.40 (m, 3H, 5-H, 6-H, 8-H), 6.25 (s, 1H, 3-H), 3.47 (s, 2H, 1′-H), 2.52 (s, 3H, 4-CH_3), 2.30 (s, 3H, 3′-H).

3,5-Dimethyl-7*H*-furo[3,2-g]chromen-7-one (**9**). Preparation of **9** followed the procedure described by MacLeod [28]. Yield 83%, m.p. 220–222 °C; ^{1}H NMR ($CDCl_3$) δ: 7.67(s, 1H, 5-H), 7.47 (s, 1H, 7-H), 7.40 (s, 1H, 8-H), 6.26 (s, 1H, 3-H), 2.51 (s, 3H, 6-CH_3), 2.27 (s, 3H, 4-CH_3).

3-(Bromomethyl)-5-methyl-7*H*-furo[3,2-g]chromen-7-one (**10**). A mixture of **9** (25.0 mmol), 1,3-dibrom-5,5-dimethylhydantoin, catalytic amount of benzoyl peroxide, and carbon tetrachloride was refluxed for 8 h. After cooling, the solvent was evaporated under reduced pressure. The residue was washed with water, purified by column chromatography (toluene/EtOAc, 100:1) and crystallized from MeOH to give **10**. Yield 25%, m.p. 183–185 °C; ^{1}H NMR ($CDCl_3$) δ: 7.58–7.30 (m, 3H, 5-H, 7-H, 8-H), 6.27 (s, 1H, 3-H), 3.10 (s, 2H, 6-CH_2Br), 2.51 (s, 3H, 4-CH_3).

5-Methyl-2-(phenylamino)-7*H*-furo[3,2-g]chromen-7-one (**11a**). A solution of **10** (10.0 mmol) in CH_3CN was mixed with powdered KOH (11.0 mmol) under N_2. Aniline was added to the mixture at 50 °C and the mixture was vigorously stirred. After 10 h, the resulting mixture was poured into water and extracted with EtOAc (20 ml × 3). The organic layer was dried over $MgSO_4$, concentrated and purified by column chromatography (hexane/EtOAc/TEA, 16:2:1) to give **11a**. Yield 72%, m.p. 203–205 °C; ^{1}H NMR (DMSO-d_6) δ: 7.53–7.21 (m, 3H, 5-H, 7-H, 8-H), 7.12–6.86 (m, 5H, 2′-H, 3′-H, 4′-H, 5′-H, 6′-H), 6.25 (s, 1H, 3-H), 2.85 (d, 2H, J = 8.0 Hz, 4-CH_2), 2.38 (s, 3H, 4-CH_3). C, H, N for $C_{19}H_{15}NO_3$.

8. Biology

8.1. Inhibition of AChE and BuChE

The inhibitory activities against AChE and BuChE of the prepared compounds were carried out using a reported method [22]. Five different concentrations of each compound were used to measure the inhibitory activity for AChE and BuChE. The assay solution consisted of 0.1 M phosphate buffer (pH 8.0), containing of 340 μM of 5,5′-dithio-bis(2-nitrobenzoic acid), 0.035 unit ml^{-1} AChE or BuChE (0.5 and 3.4 UI mg^{-1}, respectively; Sigma Chemical), and 550 μM acetylthicoline iodide or butylthicoline iodide. Test compound were added to the assay solution and pre-incubated with the enzyme for 20 min followed by the addition of substrate. Assays were carried out with a blank containing all components except AChE or BuChE in order to account for non-enzymatic reaction. The reaction rates were compared, and the percent inhibition due to the presence of test compounds was calculated. Each concentration was analyzed in triplicate, and IC_{50} values were determined graphically from log concentration-inhibition curves.

8.2. Kinetic characterization of AChE inhibition

Kinetic characterization of AChE were performed using a reported method [29]. Five different concentrations of AChE and inhibitors (**6b**, **7b**, propidium and tacrine) were mixed in the assay buffer (pH 8.0), containing of 340 μM of 5,5′-dithio-bis(2-nitrobenzoic acid), 0.035 unit ml^{-1} AChE, and 550 μM acetylthicoline iodide. Test compound were added to the assay solution and pre-incubated with the enzyme at 37 °C for 20 min, followed by the addition of substrate. The determination of kinetic characterization of the AChE-catalyzed hydrolysis of acetylthiocholine was taken by spectrometrically at 412 nm. A parallel control with no inhibitor in the mixture, allowed adjusting activities measured at various time.

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