Brief Articles

Extensive SAR and Computational Studies of 3-{4-[(Benzylmethylamino)methyl]phenyl}-6,7-dimethoxy-2*H*-2-chromenone (AP2238) Derivatives

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AP2238 was the first compound published to bind both anionic sites of the human acetylcholinesterase, allowing the simultaneous inhibition of the catalytic and the amyloid-β pro-aggregating activities of AChE. Here we attempted to derive a comprehensive structure−activity relationship picture for this molecule, affording 28 derivatives for which AChE and BChE inhibitory activities were evaluated. Selected compounds were also tested for their ability to prevent the AChE-induced Aβ-aggregation. Moreover, docking simulations and molecular orbital calculations were performed.

Introduction

Alzheimer's disease (AD[a]),[1] the most common form of dementia, was described for the first time a century ago by Alois Alzheimer. He described typical clinic characteristics such as memory disturbance and the neuropathological picture with miliary bodies and dense bundles of fibrils, which are known today as plaques and tangles, the hallmarks of the disease. The cholinergic hypothesis in AD states that degeneration of cholinergic neurons in the basal forebrain nuclei causes disturbances in presynaptic cholinergic terminals in the hippocampus and neocortex, which could account for memory alterations and other cognitive symptoms.[2] One therapeutic approach to enhance cholinergic neurotransmission is the increase in acetylcholine (ACh) availability induced by inhibiting acetylcholinesterase (AChE). Besides its catalytic function, AChE also acts as a promoter of Aβ fibril formation, this effect being independent from its normal hydrolyzing activity and associated with the peripheral anionic site (PAS) of the enzyme.[3] This finding stimulated a great interest toward the design of hybrid molecules capable of simultaneously inhibiting AChE and AChE-induced Aβ aggregation. Our research group has been involved in the development of AChE inhibitors for several years, and recently, this dual target approach has been addressed by linking a benzylamino group and a coumarin heterocycle through a phenyl ring. The combined molecule, **1** (AP2238), proved to be able to simultaneously contact both the central and the peripheral anionic sites of AChE.[4] In this paper, we intend to extensively investigate the structure−activity relationships (SARs) of **1**. We modified the lead compound **1** in all its peculiar parts (Chart 1): the coumarin moiety and its substituents, the position of the benzylamino group with respect to the coumarin nucleus, and the benzylamino moiety itself. A total of 28 analogues (Table 1) of **1** were synthesized, and their biological activities were tested using the hAChE and BChE enzymes. Eight compounds, properly selected, were also tested for their ability to prevent the AChE-induced Aβ aggregation. In addition, docking simulations and molecular orbital calculations were carried out to study the binding mode of the inhibitors at the hAChE gorge.

Chemistry

The synthesis of the compounds **1**−**29** is reported in Scheme 1. The previously synthesized intermediates **30**−**44** were brominated using *N*-bromosuccinimide in CCl_4 with a catalytic amount of $(PhCOO)_2O$ to give the bromo-derivatives **45**−**59**. The nucleophilic attack of the selected benzylalkylamine on the bromo-derivatives afforded the compounds **1**−**18** and **20**−**28**. Methylation of compound **1** with CH_3I gave **29**. Reduction of compound **18** with H_2 over $Pd/CaCO_3$ gave **19**.

Syntheses of heterocyclic intermediates **30**−**44** are reported in Schemes 2−5 (see Supporting Information (SI)).

Results and Discussion

Evaluation of Structure−Activity Relationships (SARs) Related to Catalytic hAChE Activity. The inhibitory potencies, expressed as IC_{50} values, of compounds **2**−**29** on recombinant hAChE and BChE from human serum in comparison with the reference **1** are reported in Table 2. The selectivity toward AChE versus BChE was, for all compounds, of 3 orders of magnitude or more. In view of a clearer presentation of the SAR of the series, five structural features of **1** were identified:

(a) Position of the Benzylalkylamino Moiety. Compound **2**, in which the *N*-benzyl-*N*-methyl group is in the *meta*-position with respect to the coumarin moiety, showed a lower AChE inhibitory potency compared to the lead **1**. The same trend was shown for monomethoxy derivatives, **3**−**6**, in which the *meta*-analogues **3** and **5** had a higher IC_{50} than the *para*-analogues **4** and **6**.

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[a] Abbreviations: SAR, structure−activity relationships; AChE, acetylcholinesterase; hAChE, human acetylcholinesterase; BChE, butyrylcholinesterase; Aβ, beta-amyloid peptide; AD, Alzheimer's disease; PAS, peripheral anionic site; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; RMSD, root mean square deviation; DFT, density functional theory; NMR, nuclear magnetic resonance.

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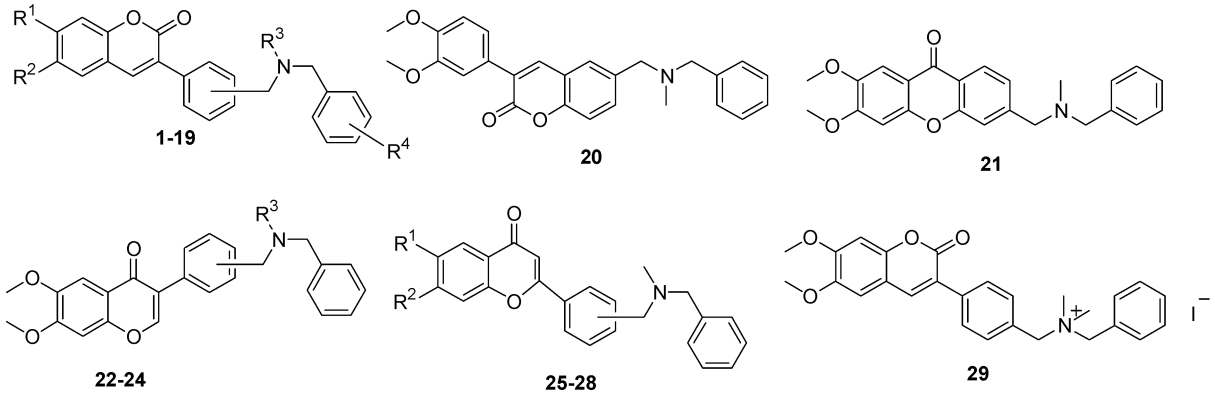
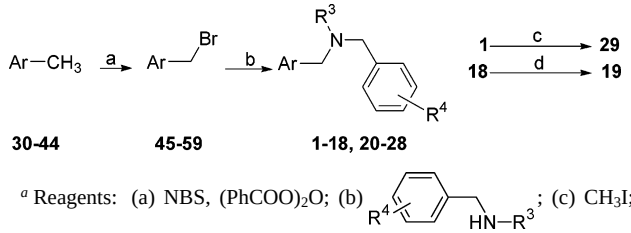
Chart 1. SAR Studies of **1** (AP2238)

Table 1. Synthesized Compounds



cpd	chain	R^1	R^2	R^3	R^4	cpd	chain	R^1	R^2	R^3	R^4
1	para	OCH_3	OCH_3	CH_3	H	**14**	para	OCH_3	OCH_3	CH_3	*m*-OCH_3
2	meta	OCH_3	OCH_3	CH_3	H	**15**	para	OCH_3	OCH_3	CH_3	*p*-OCH_3
3	meta	OCH_3	H	CH_3	H	**16**	para	OCH_3	OCH_3	C_2H_5	H
4	para	OCH_3	H	CH_3	H	**17**	para	OCH_3	OCH_3	C_2H_5OH	H
5	meta	H	OCH_3	CH_3	H	**18**	para	H	NO_2	CH_3	H
6	para	H	OCH_3	CH_3	H	**19**	para	H	NH_2	CH_3	H
7	para	OCH_3	OCH_3	CH_3	*o*-NO_2	**22**	para			CH_3	
8	para	OCH_3	OCH_3	CH_3	*m*-NO_2	**23**	meta			CH_3	
9	para	OCH_3	OCH_3	CH_3	*p*-NO_2	**24**	meta			C_2H_5	
10	para	OCH_3	OCH_3	CH_3	*o*-CH_3	**25**	meta	OCH_3	OCH_3		
11	para	OCH_3	OCH_3	CH_3	*m*-CH_3	**26**	para	OCH_3	OCH_3		
12	para	OCH_3	OCH_3	CH_3	*p*-CH_3	**27**	para	OCH_3	H		
13	para	OCH_3	OCH_3	CH_3	*o*-OCH_3	**28**	para	H	OCH_3		

Scheme 1[a]



[a] Reagents: (a) NBS, $(PhCOO)_2O$; (b) ; (c) CH_3I; (d) H_2, $Pd/CaCO_3$. [b]Number sequence of compounds is referred to Table 1 and Schemes 2−5 (see SI).

(b) Substituents on the Heteroaromatic Nucleus. Considering the methoxy groups in positions 6 and 7, for both *meta*- and *para*-compounds, the substitution in position 6 seemed to be more important than the substitution in position 7 to maintain a submicromolar activity. On the basis of previous results, we introduced an electron-withdrawing (NO_2) and an electron-donating (NH_2) substituent in position 6 of the coumarin moiety with the *N*-benzyl-*N*-methyl group in *para*-position (compounds **18** and **19**, respectively). The IC_{50} of both compounds was higher than the IC_{50} of the lead compound, but similar to the 6-monomethoxy derivative **6**.

From the analysis of points (**a**) and (**b**) of the SAR study, it clearly emerged that the *N*-benzyl-*N*-methyl group in *para*-position and the 6,7-dimethoxy coumarin conferred the best anticholinesterase activity so far (see Table 2).

(c) Substituents on the Terminal Phenyl Ring. On the basis of previous results, we maintained the dimethoxycoumarin with the benzylamino group in *para*-position and introduced both electron withdrawing and electron-donor substituents on the terminal phenyl ring. *Ortho*-, *meta*-, and *para*-nitro (**7**−**9**), -methyl (**10**−**12**), and -methoxy (**13**−**15**) derivatives were synthesized. All these compounds, with the exception of **13** (*o*-methoxy), showed a reduced anticholinesterase activity, probably indicating that the unsubstituted *N*-benzyl group completely filled the bottom of the AChE gorge and that no increase in hindrance on this part of the molecule was allowed. Exceptionally, compound **13** showed an IC_{50} that is of the same order of magnitude as the lead compound **1**. This could be explained, as seen for other AChE inhibitors,[5−7] considering that the *o*-methoxy group is able to stabilize the formal positive charge of the amino group through mixed inductive and mesomeric effects,[8] which is crucial not only to properly interact with the central aromatic residue by cation−π interaction, but also to induce the molecule to penetrate the gorge.[9]

(d) Substituent on the Basic Nitrogen. To confirm the importance of the positive charge, the methyl ammonium analogue of **1** (**29**) was synthesized and showed the lowest IC_{50} of the series. We also replaced the methyl group of the amino function of **1** with ethyl and hydroxyethyl groups, affording **16** and **17**, respectively. Compound **16**, showing an IC_{50} value of 18.3 nM, is more than 2 times more active than compound **1**. This result was in agreement with data presented by other authors,[5,6] where the introduction of an ethyl substituent conferred a higher potency. The better activity could be related to the increased lipophilicity of compound **16** compared to that

Table 2. Inhibition of AChE and BChE Activities and AChE-mediated Aβ-aggregation

cpd	IC_{50} (nM) AChE ± SEM	IC_{50} (nM) BChE ± SEM	inhibition of Aβ(1−40) aggregation[b] SEM within 5%	cpd	IC_{50} (nM) AChE ± SEM	IC_{50} (nM) BChE ± SEM	inhibition of Aβ(1−40) aggregation[b] SEM within 5%
1	44.5 ± 6.5	48.9×10^3	35	**16**	18.3 ± 3.2	118×10^3	38
2	202 ± 9.0	279×10^3	25	**17**	49.3 ± 2.3	35.1×10^3	6
3	2290 ± 290	$>10^{-5}$		**18**	148 ± 14	$>10^{-5}$	<5
4	892 ± 54	570×10^3	<5	**19**	281 ± 13	$>10^{-5}$	<5
5	303 ± 9.0)	136×10^3		**20**	not solub	not solub	
6	163 ± 7.0	1300×10^3	<5	**21**	30 300	$>10^{-5}$	
7	987 ± 76	$>10^{-5}$		**22**	1230 ± 60	$>10^{-5}$	
8	368 ± 23	$>10^{-5}$		**23**	2020 ± 180	$>10^{-5}$	
9	24 600	$>10^{-5}$		**24**	1590 ± 60	$>10^{-5}$	
10	366 ± 40	$>10^{-5}$		**25**	2270 ± 270	$>10^{-5}$	
11	122 ± 40	$>10^{-5}$		**26**	3100 ± 390	$>10^{-5}$	
12	310 ± 36	$>10^{-5}$		**27**	18 100	88.9×10^3	
13	62.1 ± 1.9	52.9×10^3	15	**28**	80 500	$>10^{-5}$	
14	714 ± 39	$>10^{-5}$		**29**	10.5 ± 5.0	43.8×10^3	
15	2260 ± 60	$>10^{-5}$					

[a] Human recombinants AChE and BChE from human serum were used. IC_{50} values represent the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of two independent measurments, each performed in triplicate (SEM = standard error of the meaning). [b] Determined at [inhibitor] = 100 μM, [Aβ(1−40)] = 230 μM and [AChE] = 2.3 μM.

of compound **1**. This hypothesis could also explain the inhibitory potency of compound **17**, which is very similar to that of **1**: the positive lipophilic effect due to two carbon units is counterbalanced by the lower lipophilicity caused by introducing the OH group.

(e) Oxygenated Heteroaryl Moiety. We inverted the position of the coumarin and the central phenyl ring of the lead compound so that the 1,2-dimethoxyphenyl moiety could interact with the PAS and the coumarin could act as a spacer; unfortunately, this compound (**20**) was insoluble, and no biological activity could be measured. The substitution of the coumarin nucleus with other oxygenated heteroaryl nuclei was performed: the 3-benzyl-6,7-coumarin was substituted with 2,3-dimethoxyxanthen-9-one leading to compound **21**, which is 3 orders of magnitude less active than **1**, probably because it is too short compared to the lead compound. The coumarin moiety was also substituted with isoflavone and flavone (compounds **22**−**24** and **25**−**28**, respectively). For all compounds, the inhibitory activity toward AChE was lower than for the reference compound **1**, and even if the dimethoxy compounds were better inhibitors than the monomethoxy derivatives, the significant decrease in their activities could not be explained by means of classical SARs.

Docking Simulations and Molecular Orbital Calculations. To thoroughly investigate the SARs of the present series of AChE inhibitors, docking simulations were carried out with the software GOLD[10] and outcomes were rationalized by means of the clustering algorithm AClAP.[11,12] Compound **1**, previously docked by means of a different procedure,[4] was studied according to the present computational strategy (see SI). Notably, the binding mode of **1** at the gorge of hAChE was very similar to that previously reported,[4] with an overall RMSD for the nonhydrogen atoms less than 1 Å. In Figure 1A, the binding mode of **1** and **2** (the *meta*-analogue of **1**) at the hAChE gorge is reported. It can be seen that while **1** could favorably interact with both the catalytic site and the PAS of the enzyme,[4] **2** was not able to properly reach Trp286. In particular, while the benzylamino moieties of both compounds could interact with Trp86 of the catalytic pocket, only the coumarin moiety of **1** could also establish a favorable $\pi-\pi$ stacking with the PAS residue Trp286. Moreover, **2** also lost the H-bond interaction with the backbone of Phe295, and this could likely explain its decreased activity when compared to **1**.

In Figure 1B, the binding mode of **22** and **26** (i.e., the isoflavone and flavone *para*-derivatives) is reported along with **1**. In this case, we observed that all inhibitors could simultaneously interact with both sites of the enzyme, and that while **22** lost the H-bond with Phe295 backbone, **26** kept such an interaction. The experimental results reported in Table 2 showed that **22** and **26** were almost 2 orders of magnitude less potent than **1**, and therefore, we had to conclude that docking outcomes alone were not able to explain the reduced potencies of **22** and **26** when compared to **1**.

To provide a possible rationalization for the SARs of these compounds, molecular orbital calculations within the density functional theory (DFT) framework were performed. The calculations were carried out on the 5,6-dimethoxycoumarin and 6,7-dimethoxychromone skeletons. In particular, we computed the energy of the frontier molecular orbital LUMO (i.e., the lowest unoccupied molecular orbital) of both scaffolds at the B3LYP/6-31G* level of theory. The results showed that the LUMO energy of 5,6-dimethoxycoumarin was almost 9 kcal/mol less than the LUMO energy of the 6,7-dimethoxychromone. Furthermore, we also carried out single-point calculations by adding one electron to both scaffolds (see SI). These calculations showed that the 5,6-dimethoxycoumarin was more prompted to accept the additional electron when compared to the 6,7-dimethoxychromone, with the energy difference being 10.3 kcal/mol in favor of the coumarin compound. These results showed the ability of the 5,6-dimethoxycoumarin moiety of **1**, which was to better accept electrons when compared to the 6,7-dimethoxychromone of **22** and **26** and therefore explained the higher inhibitory potency of **1** relative to **22** and **26** toward the hAChE enzyme. Actually, it is now widely accepted that Trp286 of the AChE PAS attracts ACh from the solvent bulk by means of cation$-\pi$ interaction,[9] demonstrating that this amino acid preferably donates (rather than accepts) electrons in $\pi-\pi$ stacking with ligands.[13] The above-reported results agree with similar computations carried out on a series of xanthostigmine derivatives,[14] demonstrating that the energy of the frontier orbitals should be taken into account when designing ligands binding at tryptophan residues.

AChE-Induced Aβ Aggregation Inhibition. Compound **1** proved to be able to simultaneously contact both the central and the peripheral anionic sites of hAChE[4] and cause inhibition of 35% in Aβ (1−40) aggregation. Inhibitors of Aβ oligomer-

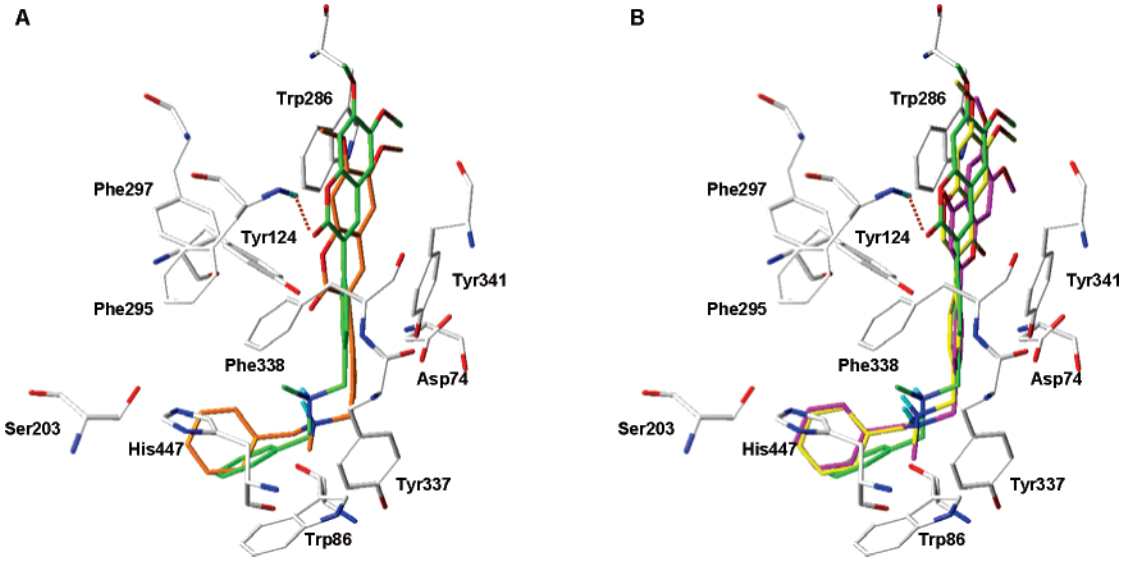


Figure 1. (A) Docking model of **1** (carbon atoms in green) and **2** (carbon atoms in orange) at the hAChE gorge. The H-bond between the carbonyl of the coumarin moiety of **1** and the hydrogen (cyan) of the Phe295 backbone NH is shown as a dark-red dotted line. It can be seen that while **1** simultaneously contacts both sites of the enzyme gorge, **2** is not properly able to reach Trp286 of the PAS. (B) Docking model of **1** (carbon atoms in green), **22** (carbon atoms in yellow), and **26** (carbon atoms in magenta) at the hAChE gorge. The same H-bond as reported in (A) is shown. It can be seen that all derivatives are able to simultaneously contact both sites of the hAChE gorge. The activity difference should, therefore, be looked for at the electronic rather than at the geometrical level.

ization have become increasingly popular as promising pharmacotherapy for AD. However, assaying many compounds for inhibition of Aβ aggregation can be a very expensive endeavor in light of the large quantities of enzyme and peptide needed for screening.[15] Consequently, for this assay we were forced to choose a limited number of compounds on the basis of their inhibitory potency. Thus, because the coumarin nucleus is supposed to interact with the PAS, some coumarins endowed with different substituents on the heterocyclic nucleus were selected. In particular, compounds **4** and **6**, with one methoxy group in positions 6 and 7, respectively, and compounds **18** and **19**, with an electron-withdrawing (NO_2) and an electron-donating (NH_2) substituent in position 6 of the coumarin moiety, respectively, were chosen. We also tested compound **2**, carrying the benzylmethylamino group in the *meta*-position, to evaluate the effect of this modification on Aβ aggregation. Moreover, compounds **13**, **16**, and **17**, being the best AChE inhibitors of the series, were also evaluated. The percentage of inhibition of AChE-induced Aβ aggregation at 100 μM is reported in Table 2. It may be noticed that the inhibitor concentration in the aggregation assay is much higher than the IC_{50} values of the same compounds. Nevertheless, as was pointed out elsewhere,[16] if "normalized" on the basis of the different amount of enzyme used in the Ellman's and aggregation assays, the ratio [inhibitor]/[AChE] is of the same magnitude in both assays. Consequently, it seems plausible that similar amounts of inhibitor can simultaneously carry out both the anti-cholinesterase and anti-aggregating actions. Most of the tested compounds did not cause any significant inhibition of Aβ aggregation, apart from **16**, where the part of the molecule interacting with the PAS is unaffected, which showed inhibition similar to that of **1** (38 and 35%, respectively). Surprisingly, compound **17**, which bears an ethanol substituent on the nitrogen interacting with the internal anionic site residues, showed a remarkably low inhibitory activity toward the AChE-induced Aβ aggregation. A possible explanation could be that the presence of a hydroxy group drives the ligand to interact by means of H-bonding with tyrosines of the AChE gorge, providing a different binding mode, which is likely unable to inhibit the pro-aggregating effect ofAChE.

Conclusions

Starting from the dual AChE inhibitor **1**, we evaluated the SAR of 28 analogues in which the modifications performed involved the substituents on the coumarin nucleus, the coumarin nucleus itself, the relative position of the benzylmethylamino group, the alkyl function on the amino group, and the substituents on the terminal phenyl ring. The few compounds showing an IC_{50} of the same order of magnitude of **1** were **13**, **16**, **17**, and **29**, while **16** (AP2243) was twice as potent as the lead compound. All these derivatives maintained the peculiar features of **1**, namely, the dimethoxy coumarin nucleus and the benzylalkylamino group in the *para*-position. Thus, the structure of **1** seems to be crucial for optimal activity, because only the modification performed on compound **16**, an ethyl instead of a methyl group on the basic nitrogen, led to an improvement in both catalytic and AChE-induced Aβ aggregation activities.

Experimental Section

Chemistry. 3-{4-[(Benzylmethylamino)methyl]phenyl}-6,7-dimethoxychromen-2-one (1). A mixture of **45** (1.32 g, 3.52 mmol) and benzylmethylamine (0.92 mL, 7.04 mmol) in toluene (125 mL) was stirred under reflux for 20 h. The solution was washed with water and extracted with 6 N HCl; the aqueous layer was basified with Na_2CO_3, and extracted with CH_2Cl_2. The combined organic layers were dried and concentrated under reduced pressure to give an oil. ^{1}H NMR ($CDCl_3$): δ 2.42 (s, 3H), 3.68 (s, 2H), 3.69 (s, 2H), 3.93 (s, 3H), 3.95 (s, 3H), 6.84 (d, 2H), 7.19−7.48 (m, 7H), 7.63 (d, 2H), 7.77 (s, 1H). The product was then converted in the hydrochloride salt affording **1**·HCl (90 mg, 6% yield): mp 230−232 °C (MeOH). ES-MS *m/z*: 452 (M + 1). Anal. ($C_{26}H_{26}ClNO_4$) C, H, N.

6-Amino-3-{4-[(benzylmethylamino)methyl]phenyl}-chromen-2-one (19). A solution of **18** (2.62 g, 6.55 mmol) in THF (250 mL) was hydrogenated in the presence of Pd/$CaCO_3$ at atmospheric pressure and room temperature. The mixture was filtered and the

filtrate was evaporated to give a residue that was purified by gradient flash chromatography. Eluting (from CH_2Cl_2 100% to CH_2Cl_2/EtOH 98:2) afforded **19** (1.7 g, 70% yield) as a dark yellow solid: mp 166−167 °C. ^{1}H NMR ($CDCl_3$): δ 2.22 (s, 3H), 3.57 (s, 2H), 3.58 (s, 2H), 6.73 (d, 1H), 6.84 (dd, 1H), 7.14−7.32 (m, 8H), 764−7.70 (m, 3H). ES-MS *m/z*: 371 (M + 1). Anal. ($C_{24}H_{22}N_2O_2$) C, H, N.

Benzyl-[4-(6,7-dimethoxy-2-oxo-2*H*-chromen-3-yl)benzyl]dimethyl Ammonium Iodide (29). A solution of CH_3I (54 mg, 0.38 mmol) and **1**·HCl (85 mg, 0.19 mmol) in MeOH (25 mL) was stirred overnight and then poured into diethyl ether. The filtered solid afforded **29** (51 mg, 24% yield) as a light beige solid: mp 145−147 °C. ^{1}H NMR (CD_3COCD_3): δ 3.26 (s, 6H), 3.88 (s, 3H), 3.98 (s, 3H), 5.14 (s, 2H), 5.17 (s, 2H), 7.02 (s, 1H), 7.28 (s, 1H), 7.23−7.62 (m, 3H), 7.75−7.78 (m, 4H), 7.91−7.98 (m, 2H), 8.22 (s, 1H). ES-MS *m/z*: 558 (M + 1). Anal. ($C_{27}H_{28}INO_4$) C, H, N, I.

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Supporting Information Available: Schemes 2−5; experimental details for intermediates **30**−**70** and final compounds **2**−**18** and **20**−**28**; computational chemistry and biological evaluation methods; and elemental analyses and ^{13}C NMR of final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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