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Article

Determining Essential Requirements for Fluorophore Selection in Various Fluorescence Applications Taking Advantage of Diverse Structure−Fluorescence Information of Chromone Derivatives

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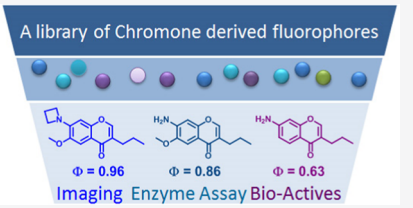
ABSTRACT: Herein, we report our work exploring the essential requirements for fluorophore selection during the development of various fluorescence applications. We assembled a library of chromone-derived fluorophores with diverse structure−fluorescence properties, which allowed us to choose the fluorophore pairs with similar structures but differing fluorescence properties and compared the performance of the selected fluorophore pairs in three types of commonly used fluorescence applications. We found that the selection standard of a suitable fluorophore is variable depending on the application. (1) In fluorescence imaging, fluorophores with strong and constant fluorescence under various conditions, such as a large pH range, are preferred. Notably, (2) in the detection of bioactive species, fluorophores with relatively lower fluorescence quantum yield favor the detection sensitivity. Furthermore, (3) in enzymatic assays employing fluorescence, the key parameter is the binding affinity between the fluorophore and the enzyme.



INTRODUCTION

Fluorescence has been widely used in various bioimaging and bioanalysis arenas.[1−9] In the majority of these studies, the fluorophore is employed as a labeling tag.[6,7] However, in recent years, reaction-based fluorescent probes have become popular and have been developed to detect various bioactive species.[5,8,10] Furthermore, the reaction-based probes have shown success in the development of sensitive fluorescence-based enzymatic activity assays.[11−15] In all of these applications, one of the key elements is, unsurprisingly, the selection of a feasible fluorophore for the system under investigation.[16,17] Fluorophores are a class of chemical compounds that emit fluorescence upon excitation.[1,2,18,19] To date, a limited number of fluorescent scaffolds have been described, including coumarin, boron-dipyrromethene (BODIPY), rhodol, and naphthalimide, and these are often used in biological and medical research.[17] It is clear that the distinct chemical structures of fluorophores grant them a range of photophysical properties, metabolic characteristics, and chemical reactivity.[3,16,20−23]

Although various fluorescence applications have been rapidly developed, there has been no general guide to help researchers choose a feasible fluorophore for their specific applications.[16,24,25] Fluorophore selection relies heavily on the experience of the researchers involved, which does not aid in their more widespread use. When selecting a fluorophore for use in experiments, the first aspect to consider is a fluorophore's specific photophysical properties.[3,7] Additionally, careful analysis of the metabolic character is required when the fluorophore is to be used in in vivo applications.[16,26] Furthermore, its inherent chemical reactivity allows chemists to rationally design the reaction-based fluorescent probes, thus providing a new avenue to investigate interesting bioactive species.[8] However, with the increased demand from the life science industry, researchers expect the fluorescence application with high selectivity, sensitivity, and accuracy.[27] As the key element of the system, it is necessary to select the most suitable fluorophore to develop fluorescence applications with good performance.

Many natural products containing the flavone and isoflavone scaffolds have been reported to exhibit fluorescence, albeit with low fluorescence quantum yields.[17,28−33] Efforts have therefore focused on the development of chromone-derived fluorescent scaffolds with improved fluorescence. Luthman and co-workers demonstrated that 3-hydroxyflavone-derived fluorophores with improved fluorescence quantum yield could be used in various applications (Φ = 0.48 in EtOH).[32] Our group have a long-standing interest in this area, and we recently described the development of a new fluorescent moiety, 3-alkyl-6-methoxy-7-hydroxy-chromone (AMHC), which exhibited a medium fluorescence quantum yield (Φ = 0.48 in 0.1 M Tris−HCl

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buffer, pH 8.0) starting from the readily available natural product 7-hydroxyisoflavone (Figure 1).[17]

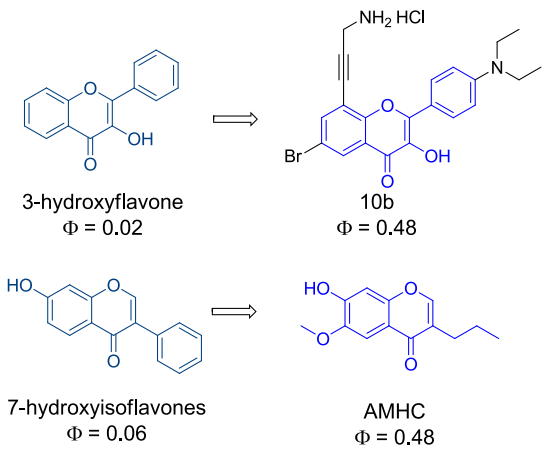


Figure 1. Previous development of 3-hydroxyflavone and 7-hydroxychromone as the interesting fluorophores.[17,32]

To ascertain the most favorable properties for a specific fluorescence application, we need to compare the performance of different fluorophores. Instead of randomly comparing fluorophores with very different fluorescence properties and physicochemical properties, such as coumarin vs BODIPY, it is more useful to provide an accurate assessment using fluorophore pairs with similar structures but one different fluorescence property. Thus, in this study, we designed and synthesized a library of interesting chromone-derived fluorophores with diverse fluorescence properties. Taking advantage of the structure−fluorescence information, we were able to select the ideal fluorophore pairs to carry out various fluorescence experiments.

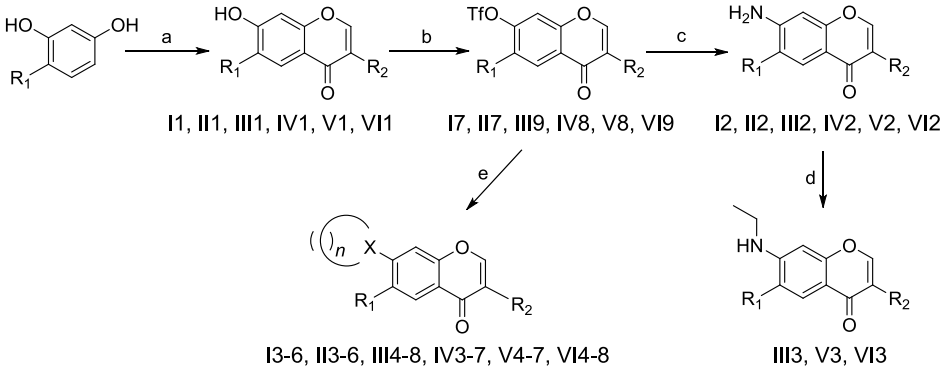
RESULTS AND DISCUSSION

Synthesis of Various Chromone-Derived Fluorophores and Fluorescent Probes. Chromone-derived fluorophores were synthesized following Scheme 1. The first step was to construct the key 7-hydroxychromone intermediate via a Friedel−Crafts acylation of the corresponding resorcinol derivatives and substituted acetic acid in the presence of a Lewis acid ($BF_3{\cdot}OEt_2$). Then, 2,4,6-trichloro-1,3,5-triazine (TCT) and dimethylformamide (DMF) were added into the mixture to form a Vilsmeier−Haack type complex,[34] which was deaminated to form the desired 7-hydroxychromone. Through this procedure, various 7-hydroxychromone analogues were obtained in good yields (≥69.9%). Next, various 7-amino chromones were obtained in two more steps. The 7-hydroxyl group was first functionalized with trifluoromethanesulfonic anhydride (Tf_2O) under aqueous conditions to yield the triflate,[35−37] followed by the synthesis of the desired amine derivative. 7-Primary amine chromone analogues were obtained by removing benzophenone oxime in acidic conditions after palladium-catalyzed Buchwald−Hartwig coupling reactions of triflate with benzophenone imine. 7-Ethylamino chromone analogues were obtained by N-monoalkylation of a primary amine with alkyl halides. 7-Tertiary amine chromone analogues were synthesized by palladium-catalyzed Buchwald−Hartwig reactions of triflate with the corresponding amines in the presence of palladium acetate.[3,36]

Synthesis of fluorescent probes of AMHC-1 and 7-(azetidin-1-yl)-3-(4-azidobutyl)-6-methoxy-4*H*-chromen-4-one (AMAC-1) was carried out as described in Scheme 2. First, 7-hydroxyl chromone VI10 was synthesized as described above.[34] The halide VI10 was then azided to give 3-(4-azidobutyl)-7-hydroxy-6-methoxy-4*H*-chromen-4-one (AMHC-1). The fluorescent dye 7-(azetidin-1-yl)-3-(4-azidobutyl)-6-methoxy-4*H*-chromen-4-one (AMAC-1) was synthesized through two steps. We first reacted AMHC-1 with trifluoromethanesulfonic anhydride under aqueous conditions to yield triflate VI11,[36] followed by a palladium-catalyzed Buchwald−Hartwig reactions in the presence of palladium acetate to yield the final AMAC-1.[3]

The synthesis of fluorescent probes of AMAC-2 to AMAC-5 was carried out according to Scheme 3. 7-Amino chromone of III2/VI2 was synthesized as described above.[34] Condensation of 7-primary amine chromone with sodium nitrite and sodium azide in H_2O/HCl at 0 °C resulted in a high yield of the desired fluorescent probes: 3-alkyl-7-azido-chromone (AMAC-2) and 3-alkyl-6-methoxy-7-azido-chromone (AMAC-3).[38,39] 7-Primary amine chromone was linked to lysine derivatives (2′- and 6′-positions were protected with *tert*-butyloxy carbonyl) via a $POCl_3$/pyridine activation procedure,[40,41]

Scheme 1. Synthesis Route for Chromone-Derived Fluorophores[a]



[a]Reagents and conditions: (a) (1) acetic acid derivatives, $BF_3{\cdot}Et_2O$, 90 °C; (2) cyanuric chloride (TCT), DMF, 60 °C; (3) boiling diluted HCl. (b) Triflic anhydride, toluene, 30% aq K_3PO_4. (c) (1) Benzophenone imine, Cs_2CO_3, $Pd(OAc)_2$, 2,2′-bis(diphenylphosphino)-1,1′-binaphthyl (BINAP), toluene; (2) 2 N aqueous HCl, tetrahydrofuran (THF); (d) bromoethane, KOH, dimethyl sulfoxide (DMSO). (e) Amine, Cs_2CO_3, $Pd(OAc)_2$, BINAP, toluene.

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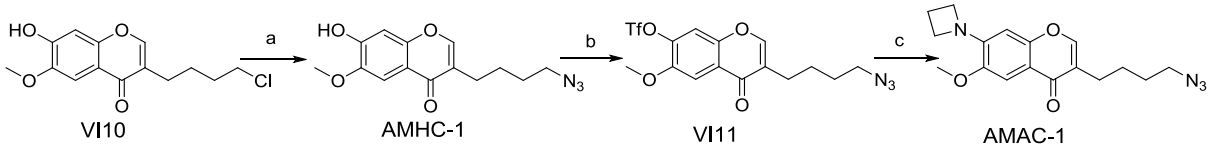
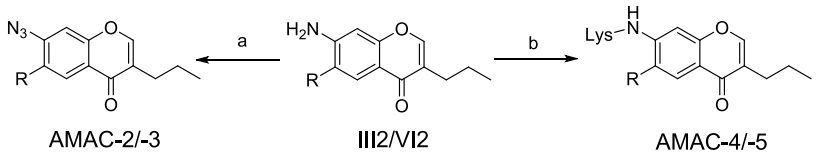
Scheme 2. Synthesis Route for AMHC-1 and AMAC-1[a]



[a]Reagents and conditions: (a) NaN_3, KOH, DMSO. (b) Triflic anhydride, toluene, 30% aq K_3PO_4. (c) Azetidine, Cs_2CO_3, $Pd(OAc)_2$, BINAP, toluene.

Scheme 3. Synthesis Route for Chromone-Derived Fluorophores[a]



[a]Reagents and conditions: (a) concentrated HCl, $NaNO_2$, NaN_3, H_2O. (b) (1) Boc-Lys(Boc)-OH, pyridine, $POCl_3$, THF; (2) 4.0 M hydrogen chloride solution in dioxane.

followed by simple deprotection under acidic conditions to yield the desired fluorescent probes 2,6-diamino-*N*-(4-oxo-3-propyl-4*H*-chromen-7-yl)hexanamide (AMAC-4) and 2,6-diamino-*N*-(6-methoxy-4-oxo-3-propyl-4*H*-chromen-7-yl)-hexanamide (AMAC-5).

The construction of chromone was previously reported with methylsulfonyl chloride as the key reagent.[17] After Friedel–Crafts acylation, the resulting reaction mixture was treated with methylsulfonyl chloride in DMF, which led to the formation of the desired 7-hydroxychromone analogues. However, the yield of this method was rather low. In this study, we optimized the synthesis of 7-chromone derivatives. The reaction mixture of Friedel–Crafts acylation was directly treated with TCT and DMF to form a Vilsmeier–Haack type complex. The final deamination will lead to 7-hydroxychromone analogues. In this protocol, we only need to pay attention to the reaction temperature. The anhydrous conditions and oxygen removal are not seriously required. Moreover, the reaction went on quickly with decent yields for a wide range of substrates.[35−37]

Fluorescence Properties of Various Chromone-Derived Fluorophores. Based on our previous experience,[17] we designed a series of six distinct chromone derivatives (Figure 2). In particular, we focused our attention on the modification

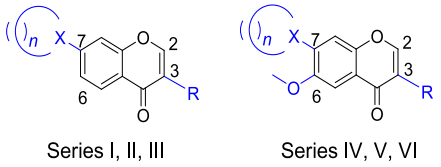


Figure 2. Exploration of the structure and fluorescence relationship of 7-donor-chromone derivatives in aqueous buffers.

of the 3- and 6-positions, as well as attaching various electron-donating groups at the 7-position. The fluorescence properties of these 40 chromone compounds were measured and are listed in Table 1.

We began by comparing the photophysical properties of 3-substituted 7-donor-chromones. In general, 3-alkyl substitution significantly increased the fluorescence quantum yield of these 7-donor-chromone than the comparative 3-aromatic substitution. For example, comparing compounds from series I, II, and III, compound I1 and I2 with a 3-toluol group has a fluorescence quantum yield of 0.08 and 0.26, respectively. In contrast, their alkyl counterparts in series II (II1 and II2) bearing a 3-methyl group has a fluorescence quantum yield of 0.21 and 0.57, respectively. Moreover, when we further analyzed the difference when employing various alkyl chains, we observed that the butyl chain is slightly better than the methyl group at increasing the fluorescence quantum yield. Interestingly, II1 and IV2 bearing a 3-methyl group have smaller fluorescence quantum yields than the 3-butyl derivatives III1 and V2.

We then moved our attention to investigating the contribution that a 6-methoxy group had toward the fluorescence of 7-donor-chromones. Interestingly, we only observed an increase in the fluorescence of 7-donor-chromone with scaffolds bearing 3-alkyl substituents, whereas no increase was observed in the corresponding 3-aryl. For example, compounds V2 and VI2 bearing a 6-methoxy group showed increased fluorescence quantum yield compared to their counterparts II2 and III2 without the 6-substitution.

Next, we examined the effect of various electron donors at the 7-position had toward the fluorescence of 7-donor-chromones. We therefore screened a wide range of possible electron donors, focusing our attention on amine and hydroxyl substituents. Generally, the amino group acted as the better electron donor compared to the hydroxyl group. In all six series, compounds with the 7-amino group (I2, II2, III2, IV2, V2, and VI2) had a higher fluorescence quantum yield than the corresponding 7-hydroxyl group (I1, II1, III1, IV1, V1, and VI1). In addition, and as expected, varying the structure of the amino groups has a direct impact on the photophysical properties. In particular, cyclized amines such as pyrrolidine and azetidine played a large role. Although most of the amino groups tested has a deleterious effect on the fluorescence, we found that 7-azetidinyl substitution (VI5) enhanced the fluorescence quantum yield of 3-alkyl-6-methoxy-chromone to 0.96 in 0.1 M Tris–HCl buffer, pH 8.0, which is the highest fluorescence quantum yield we observed for these chromone derivatives. Moreover, this compound, VI5, has outstanding brightness compared to other fluorophores in the series (Figure 3). In addition, although 7-azetidinyl substitution decreased the fluorescence quantum yield of 3-alkyl-chromone, it significantly increased the photostability of III5 with a lifetime of 12.88 ns (Table 1).

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Table 1. Photophysical Properties of Six Distinct Series of Chromone-Derived Compounds

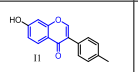
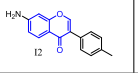
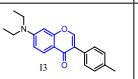
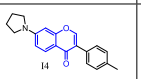
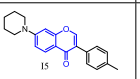
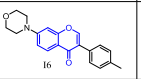
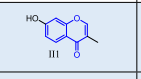
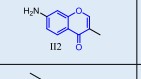
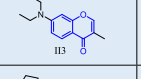
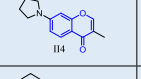
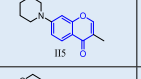
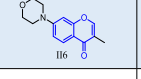
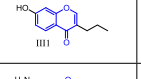
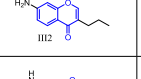
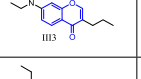
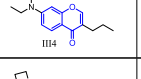
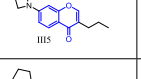
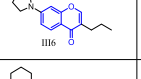
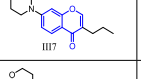
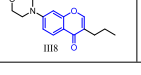
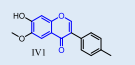
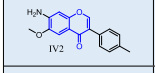
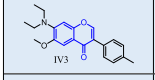
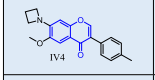
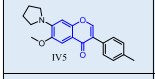
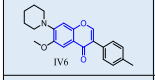
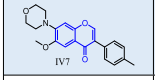
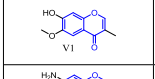
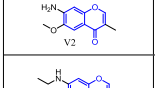
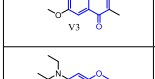
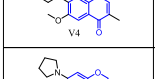
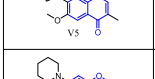
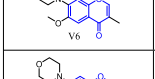
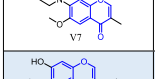
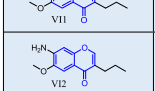
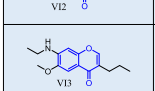
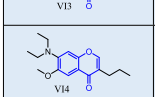
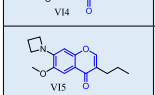
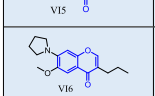
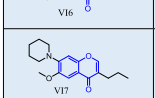
Compound[a]	ε_{max}[b]	λ_{ab}[c]	λ_{em}[c]	Φ[d]	τ[e]
	11600	334	466	0.08	
	8600	315	450	0.26	
	1200	362	481	0.02	
	2800	344	461	0.03	
	6000	332	428	0.01	
	3600	332	470	0.06	
	14100	333	457	0.21	
	11900	317	443	0.57	
	13000	332	457	0.23	
	16600	362	478	0.12	
	12000	337	485	0.02	
	13200	330	469	0.06	
	11600	332	457	0.23	2.08
	12500	317	442	0.63	1.32
	10200	337	454	0.49	1.61
	5600	355	466	0.24	1.40
	10800	337	472	0.29	12.88
	7800	361	475	0.13	4.25
	2800	340	484	0.03	1.98
	13000	330	467	0.09	

Table 1. continued

Compound[a]	ε_{max}[b]	λ_{ab}[c]	λ_{em}[c]	Φ[d]	τ[e]
	16100	344	445	0.04	
	9100	336	445	0.14	
	8900	348	446	0.01	
	7900	347	480	0.13	
	2700	346	459	0.01	
	1500	347	396	0.04	
	5500	336	473	0.01	
	17300	342	446	0.45	
	14000	334	433	0.76	
	18400	344	443	0.63	
	13800	340	441	0.01	
	14500	348	462	0.01	
	11800	345	444	<0.01	
	12900	336	450	<0.01	
	17900	343	446	0.48	
	18900	333	432	0.86	2.57
	10400	317	398	0.74	1.50
	8300	342	442	0.06	1.47
	15900	345	438	0.96	2.39
	7200	347	456	0.03	4.87
	8800	347	460	0.03	2.72
	15000	345	443	0.03	

[a]The measurements were taken in 0.1 M Tris−HCl, pH 8.0. [b]Unit $M^{-1}\cdot cm^{-1}$. [c]Unit nm. [d]Determined with quinine sulfate (Φ = 0.54, 0.1 M H_2SO_4).[42] [e]Unit ns.

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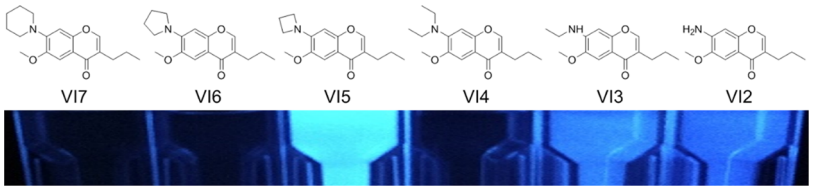


Figure 3. Fluorescence intensity of compounds of series VI with the scaffold of 3-alkyl-6-methoxy-7-amino-chromone in 0.1 M Tris−HCl buffer, pH 8.0. Fluorophore VI5 has outstanding brightness over other fluorophores.

According to the Jablonski diagram, the excited fluorophore will undergo various radiative (fluorescence) and nonradiative processes (nonradiative decay, thermal relaxation) before returning to the ground state.[19] Thus, it is difficult to accurately manipulate the balance between the radiative and nonradiative processes via structural modification of the fluorophore.[3,17,22,23,43] In this study, the optimization of the photophysical properties of 7-donor-chromone was predominantly based on "trial and error" and our previous experience working with these chromone scaffolds.[17]

7-Hydroxychromone conforms to the donor−acceptor (push−pull) fluorescence system. The 7-position serves as the donor group, with the chromone scaffold itself acting as the p-conjugated-acceptor structure.[21] We initially optimized the acceptor system via modification on various positions of the chromone core to adjust the intermolecular energy-transfer processes of the acceptor. From our previous experience,[17] the modification at 3- and 6-positions was shown to be the most important effect for the 7-hydroxychromone scaffold. Thus, in this study, we designed six distinct series of compounds with the noted changes (phenyl, methyl, butyl) on the 3-position and the changes (H and methoxy) on the 6-position. For substituents on the 3-position, we varied the alkyl and aromatic groups to elucidate the key differences. Although both alkyl and aromatic groups are weak electron donor groups, their installation on the chromone scaffold (the electron acceptor) provided differential quantum yields. 3-Alkyl substitution favors the improvement of fluorescence quantum yields of 7-donor chromones. For the 6-position, the attachment of the electron-donating methoxy substituent provided notable differences in the fluorescence quantum yields of the 7-donor chromone. An increase in the fluorescence quantum yield was also observed when both the 6-methoxy and 3-alkyl groups on the 7-donor chromone are present.

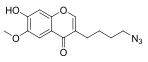
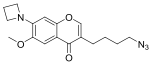
Second, we further screened various electron donors at the 7-position to find the one that provided a general improvement in the quantum yield of the chromone scaffold. In our study, we found that an amino group is preferred over a hydroxyl group as this improved the fluorescence quantum yield reproducibly. However, compared to the naked amino group, the substituted amino groups showed different effects on the fluorescence quantum yields. In general, the substitution (NHR, NRR′, cyclized amino) can prevent the rotation of the amino group, and the substitution causes the fluorophore to preferentially undergo nonradioactive decay, which results in a decrease of the fluorescence quantum yield.[19,20,43] This lower quantum effect can be explained as the formation of a twisted internal charge transfer (TICT) state.[19,20,23,43,44] Interestingly, the installation of a three or four-member ring can inhibit the TICT effect.[43] The use of small rings (aziridine and azetidine) eventually improves the fluorescence quantum yield dramatically, as well as the brightness and the overall photostability of the donor−acceptor fluorescence systems.

Taken together, we can see that all compounds (II2, III2, V2, V3, VI2, VI3, and VI5) within the 3-alkyl-7-amino-chromone family show decent fluorescence properties (Φ > 0.55 in 0.1 M Tris−HCl buffer), while the installation of the 6-methoxy group further increases the fluorescence quantum yield and fluorescence brightness of 3-alkyl-7-amino-chromone.

Study on the Fluorophore Selection in Fluorescence Imaging. Before we assessed our synthesized chromones in various fluorescence applications, four key chromone derivatives (III2, III5, VI2, and VI5) were evaluated for cell cytotoxicity to ensure that they were suitable for biological applications. We tested the four derivatives against three human cancer cell lines, HCT-116, HepG2, and BGC-823, and did not find any obvious toxicity at high concentrations (50 μM).[4] Taking advantage of the diverse structure−fluorescence information obtained for the chromone derivatives, we were able to select three pairs of fluorophores with similar structures, which had one obvious different fluorescence property. We then investigated the effect of that specific fluorescence property on three commonly used fluorescence applications: fluorescence imaging, fluorescence detection of bioactive species, and fluorescent enzymatic assays.

We chose VI1 (3-butyl-6-methoxy-7-hydroxyl-chromone) and VI5 (3-butyl-6-methoxy-7-azetidinyl-chromone) as the core fluorophores, as they both possessed impressive fluorescence intensities in aqueous solution (Table 2). We

Table 2. Photophysical Properties of Two Chromone-Derived Fluorescent Probes

Structure[a]	Cmpd	ε_{max}[b]	λ_{ab}[c]	λ_{em}[c]	Φ[d]
	AMHC-1	16300	346	446	0.48
	AMAC-1	16100	345	438	0.96

[a]The measurements were taken in 0.1 M Tris−HCl, pH 8.0. [b]Unit $M^{-1}\cdot cm^{-1}$. [c]Unit nm. [d]Determined with quinine sulfate (Φ = 0.54, 0.1 M H_2SO_4).[42]

synthesized the dyes AMHC-1 and AMAC-1 bearing a terminal azide for use in bio-orthogonal labeling (Table 2), and the fluorescence spectra were measured in aqueous solutions with various pH values. As is shown in Figure 4A, the fluorescence of AMHC-1 is pH dependent, which shows dramatically increased fluorescence intensity under basic conditions,[17] which corresponds to our previous study, wherein we reported the excellent imaging performance of AMHC-1 in a basic aqueous buffer (pH 9.0−10.0).[17] Interestingly, the fluorescence of AMAC-1 bearing a 7-azetidinyl group exhibits less pH-dependent fluorescence

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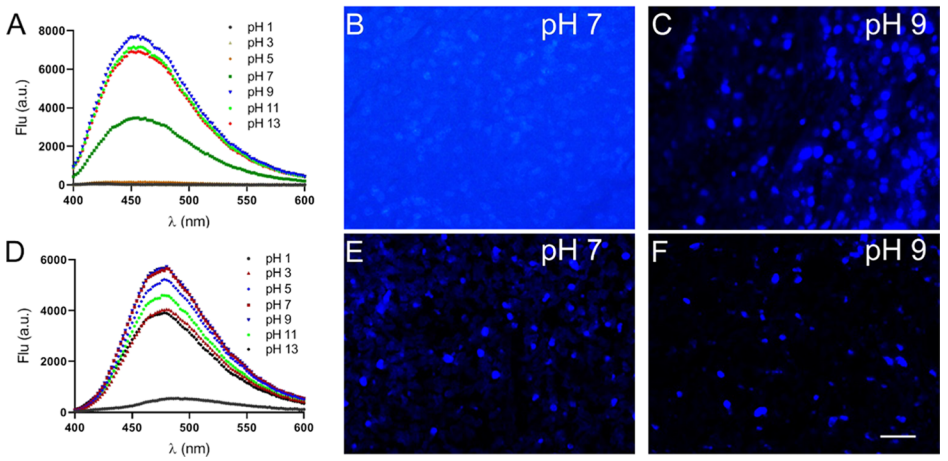


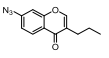
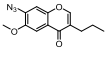
Figure 4. Fluorescence responses of AMHC-1 and AMAC-1 under various pH conditions. Fluorescence emission spectra and fluorescence intensity of AMHC-1 (A) and AMAC-1 (D) at various pH values ranging from 1.0, 3.0, 5.0, 7.0, 9.0, 11.0, and 13.0; the application of the dyes AMHC-1 and AMAC-1 to label and image 5-ethynyl-2′-deoxyuridine (EdU) that was incorporated into DNA in mice tumor tissues in phosphate-buffered saline (PBS) buffer, pH 7.0 (B, E) and PBS buffer, pH 9.0 (C, F). Scale bar indicates 50 μm.

intensity, and AMAC-1 remains relatively stable under a big range of pH values (pH 3.0−13.0) (Figure 4C). Indeed, in our fluorescence imaging experiment, in the aqueous buffer with pH 7.0, AMAC-1 (Figure 4E) showed much stronger fluorescence than AMHC-1 (Figure 4B) under similar microscopy lighting conditions. However, when we changed the aqueous buffer to pH 9.0, both dyes AMHC-1 (Figure 4C) and AMAC-1 (Figure 4F) performed well. Thus, compared to AMHC-1, the fluorophore AMAC-1 with strong and constant fluorescence intensity under a large range of pH values can achieve more robust results in biological imaging.

Fluorescence imaging is a powerful tool to investigate biological events, with fluorophores exhibiting strong fluorescence intensity playing a key role in this imaging.[3] We wanted to further explore this area and investigate what other properties are important for biological imaging. A large number of fluorophores belong to the donor−acceptor (push−pull) fluorescence system,[21] and basic conditions favor electron donors. However, most physiological environments remain relatively acidic, such as that found in the microenvironment around and in cancerous cells. In this study, although probe AMHC-1 exhibited strong fluorescence intensity under basic conditions (pH = 9.0), it does not show clear fluorescent labeling in a neutral buffer (pH = 7.0) and thus limits the application of AMHC-1 in a biological setting. Interestingly, AMAC-1 exhibits constant fluorescence under a large range of pH values (pH 3.0−13.0) and is therefore more suitable to be used as a fluorescent label in biological imaging studies. Therefore, it is important to choose the fluorophore with strong and constant fluorescence intensity across a range of pH values as fluorescent labels.

Study on the Fluorophore Selection in Reaction-Based Bioanalysis. The design and application of reaction-based fluorescent probes are one of the most popular research areas in recent years.[8] We were interested in exploring which parameters were important in selecting fluorophores for this type of application. Probes AMAC-2 and AMAC-3 were designed based on the fluorophores of III2 and VI2. The structures of AMAC-2 and AMAC-3 contained a N_3 group, which can be reduced to the amino group by the action of H_2S and, therefore, be used to directly monitor or sense H_2S in situ. Our results show that the modification of the 7-amino group clearly reduced the fluorescence quantum yields of the probes; however, we were still able to detect low-intensity fluorescence in both probes (Table 3). Moreover, the maximum

Table 3. Photophysical Properties of Two Chromone-Derived Fluorescent Probes

Structure[a]	Cmpd	ε_{max}[b]	λ_{ab}[c]	λ_{em}[c]	Φ[d]	Ratio
	AMAC-2	4900	329	443	0.01	63
	AMAC-3	3300	338	433	0.10	8.6

[a]The measurements were taken in 0.1 M Tris−HCl, pH 8.0. [b]Unit $M^{-1}\cdot cm^{-1}$. [c]Unit nm. [d]Determined with quinine sulfate (Φ = 0.54, 0.1 M H_2SO_4).[42]

fluorescence turn-on ratio can roughly be calculated as the fluorescence quantum yield ratio between the mother fluorophore and the probe. Interestingly, probe AMAC-2 displayed a lower fluorescence quantum yield but higher fluorescence turn-on ratio than the probe AMAC-3.

Once synthesized, we endeavored to test our designed probes as H_2S sensors. The sensing ability and specificity toward H_2S was investigated in 0.1 M Tris−HCl buffer at pH 8.0. Various biologically important species, such as GSH, Cys, HS^-, SO_3^{2-}, HSO_3^-, $S_2O_3^{2-}$, $S_2O_5^{2-}$, lipoic acid, HSO_4^-, SO_4^{2-}, NO_2, L-ascorbic acid, Fe^{2+}, NO, H_2O_2, *tert*-butyl hydroperoxide (TBHP), ClO^-, ClO_4^-, $^1O_2$, and $^\bullet OH$, were incubated with probes AMAC-2 and AMAC-3 and the fluorescence were measured (Figure 5). As anticipated, we only observed fluorescence when HS^- was present and saw a clear turn-on of fluorescence of both probes in 5 min (Figure 5). After 5 min incubation with the analyte, the fluorescence reached half the maximal level, while the fluorescence reached the peak level in an hour. Notably, probe AMAC-3 had a higher fluorescence background and a lower fluorescence turn-on ratio than probe AMAC-2, which restricts the H_2S detection sensitivity of AMAC-3. We calculated the limit of detection (LOD) of probes AMAC-2 and AMAC-3 toward

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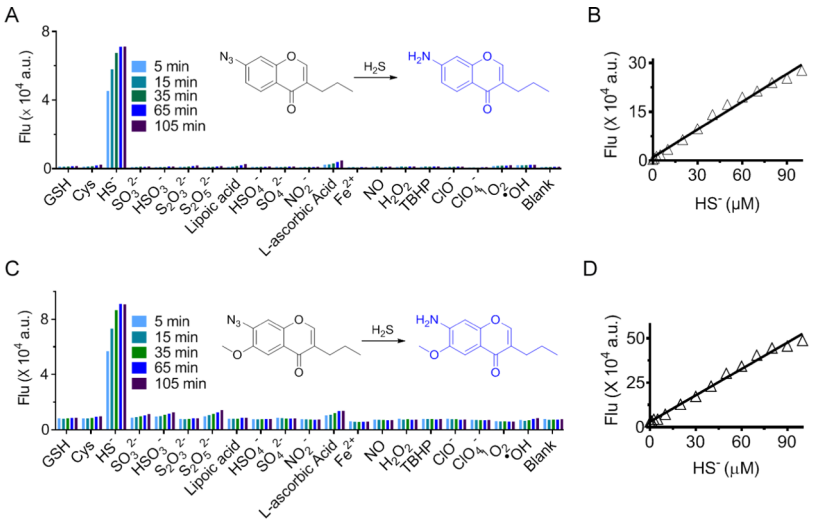


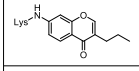
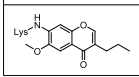
Figure 5. Application of probes AMAC-2 and AMAC-3 to detect hydrogen sulfide. The detection results of probe AMAC-2 are shown in (A) and (B), and the results of probe AMAC-3 are shown in (C) and (D). All of the measurements were acquired in 0.1 M Tris−HCl, pH 8.0 with E_x = 337 nm, E_m = 472 nm. (A) and (C) showed the detection specificity of probes AMAC-2 and AMAC-3 toward H_2S. A 10 μM probe AMAC-2 or AMAC-3 was incubated with various biological important species (500−1000 μM), and the fluorescence was measured. (B) and (D) showed that the LOD of each probe toward H_2S was also measured, various concentrations of NaHS incubated with probe AMAC-2 or AMAC-3 (10 μM) to obtain a concomitant linear increase in fluorescence.

H_2S as 0.02 and 0.03 μM, respectively. This is also correlated to the maximum turn-on ratio of these two probes (Table 2).

The application of fluorescence techniques in sensing various biologically important species is rapidly growing.[45−47] Various reaction-based fluorescent probes have been designed to specifically detect the presence of biologically important species. H_2S is an important intracellular signaling molecule, and the detection of H_2S is useful to understand the status of various biological systems.[48] Numerous H_2S recognition reactions have been used to design fluorescent probes, such as nucleophilic addition to α,β-unsaturated ketones and reduction of azides.[49−52] To detect H_2S with high sensitivity and specificity, continuous efforts are dedicated to explore new reactions. However, there is an absence of study into the effect of the specific fluorophore on the detection sensitivity. In this section, we took advantage of the previously mentioned structure−fluorescence information of our bespoke chromone-derived fluorophore and explored which aspect of the fluorophore was required to achieve good sensing sensitivity. We developed two 7-azide-substituted chromones, AMAC-2 and AMAC-3, as specific sensors of H_2S. We observed that, overall, the sensitivity and specificity of our probes are reasonable. We observed that AMAC-3 (LOD = 0.03 μM) had a lower detection sensitivity when compared to AMAC-2 (LOD = 0.02 μM). Interestingly, the detection sensitivity correlated to the maximum turn-on ratio of these two probes. Fluorophores with high fluorescence quantum yields are inherently difficult to quench using the same reactive group. This causes a high initial fluorescence background and also directly affects the overall fluorescence turn-on ratio. Thus, when selecting the fluorophore for this type of analysis, one with relatively lower fluorescence quantum yields is likely to achieve better detection sensitivity.

Study on the Fluorophore Selection in Enzymatic Assays. We then turned our attention to fluorophore selection in a fluorescent biochemical assay.[14,15] In this type of assay, the fluorophore is designed as part of the substrate of the targeted enzyme and the fluorescence is subsequently quenched via enzymatic reaction. In this study, we applied a commonly used enzyme, trypsin, which can specifically hydrolyze the amide bond after a lysine or arginine residue.[14,15] We therefore coupled a lysine residue with 7-amino fluorophores to produce probes AMAC-4 and AMAC-5, which were based on the fluorophores III2 and VI2. The newly formed amide bond partially quenched the fluorescence of the fluorophore, but probes AMAC-4 and AMAC-5 still showed low-level fluorescence with a Φ of 0.03 and 0.14 (Table 4) with probe

Table 4. Photophysical Properties of Two Chromone-Derived Fluorescent Probes

Structure[a]	Cmpd	ε_{max}[b]	λ_{ab}[c]	λ_{em}[c]	Φ[d]	Ratio
	AMAC-4	12200	308	406	0.03	21
	AMAC-5	10700	329	391	0.14	6.1

[a]The measurements were taken in 0.1 M Tris−HCl, pH 8.0. [b]Unit $M^{-1}\cdot cm^{-1}$. [c]Unit nm. [d]Determined with quinine sulfate (Φ = 0.54, 0.1 M H_2SO_4).

AMAC-4 displaying lower fluorescence quantum yield but higher fluorescence turn-on ratio than AMAC-5. We incubated the two probes with 50 U/mL of trypsin in 0.1 M Tris−HCl, at pH 8.0 buffer and, as expected, both of these probes performed well as substrates for trypsin. Interestingly, probe AMAC-4 had a K_m of 47.55 μM, while AMAC-5 had a K_m of 30.33 μM. Thus, probe AMAC-5 is more suitable as the substrate of trypsin, although AMAC-5 has a relatively small fluorescence turn-on ratio. We used computational docking to try and explain the binding mode of AMAC-4 and AMAC-5 to the active center of trypsin.[53] This showed that AMAC-4 and AMAC-5 bound in the same conformation in the active site of the enzyme (Figure 6C,D). However, the additional methoxyl group on AMAC-5 showed a weak hydrophobic interaction

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with Trp215, which was consistent with the trend in K_m between AMAC-4 and AMAC-5.

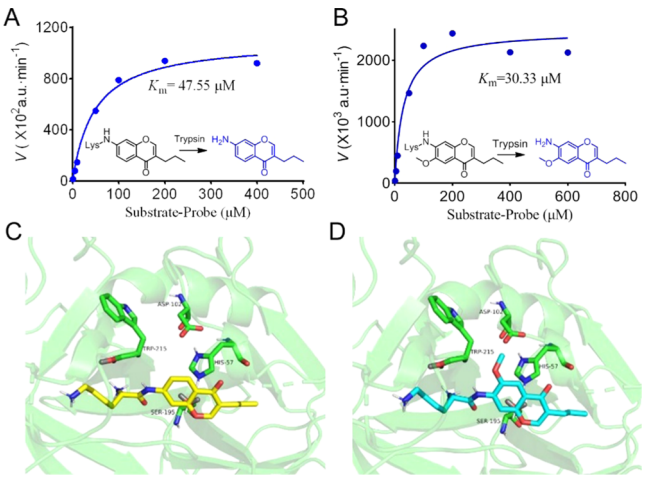

Figure 6. Enzyme kinetic study of trypsin using two substrates containing either AMAC-4 or AMAC-5. The measurements were acquired in 0.1 M Tris−HCl, pH 8.0 with E_x = 337 nm, E_m = 472 nm. Various concentrations of substrates were incubated with 50 U/mL of trypsin in 0.1 M Tris−HCl, pH 8.0 buffer, and the fluorescence was measured every 2 min to calculate reaction velocity. The kinetic parameter K_m was calculated for each substrate AMAC-4 (A) and AMAC-5 (B). In addition, the docking studies of AMAC-4 (C) and AMAC-5 (D) with trypsin (PDB code, 2A31) were also carried out.[53]

In recent years, the use of fluorophores in various biochemical analyses, particularly when measuring inhibitory profiles of small molecules, has increased dramatically. In this study, we explored the selection of fluorophores in a well-characterized biochemical assay. We used our fluorophores as a parent scaffold to construct trypsin substrates and used them to carry out a real-time kinetic study. AMAC-5 exhibited a higher initial fluorescence background and a lower fluorescence turn-on ratio than AMAC-4; however, AMAC-5 bound slightly better in the active center of trypsin. Through the use of computational docking, we showed that the additional 6-methoxyl group of AMAC-5 can interact with TRP215 and, thus, provide an insight into our experimental results. Interestingly, the potential use of fluorophores in biochemical analysis mainly depends on the binding affinity of the fluorophore to the active center of the enzyme and not the fluorescence turn-on ratio or the fluorescence quantum yields of the fluorophore. Thus, fluorescent enzymatic assays and reaction-based bioactive species detection require the fluorescent probes to have significantly different properties. In the detection of bioactive species, where the concentration of the analyte is often limited, the initial fluorescence background and fluorescence turn-on ratio are important for successful detection. In comparison, for fluorescent enzymatic assays, it is the speed of fluorescence change, which represents the activity of the enzyme. Thus, the binding between the fluorescent probe and the enzyme is much more important for the detection.

CONCLUSIONS

In this study, we synthesized and measured the fluorescence properties of six distinct series of chromone derivatives. We assembled a library of chromone derivatives with diverse fluorescence properties, and several fluorophores derived from this scaffold were shown to have impressive fluorescence quantum yields and photostability. Particularly, we chose several chromone-derived fluorophores with similar structures but different fluorescence properties to compare their effects on various biological applications. We found that different fluorescence applications have different preferences, and these must be taken into consideration when choosing appropriate fluorophores. When used in fluorescence imaging, fluorophores with strong and constant fluorescence intensity over a large range of pH values are preferred; when used to design reaction-based fluorescent sensors to detect bioactive species, the fluorophore with a relatively lower fluorescence quantum yield but a bigger fluorescence turn-on ratio can help in improving the detection sensitivity. Finally, when used in real-time fluorescent enzymatic activity assays, the high binding affinity between the fluorophore and the enzyme is required to achieve high performance. Taken together, this study provides some basic information to guide the selection of a feasible fluorophore in various biomedical studies.

EXPERIMENTAL SECTION

Synthesis of Various Chromone-Derived Fluorophores. All of the solvents and chemicals were purchased from commercial sources: Sigma-Aldrich Chemical Co., Beijing Ou-he Reagents Co., Beijing Shiji-Aoke Biotechnology Co., and Shanghai Jingke Chemistry Technology Co. with the purity of more than 95% (liquid chromatography-mass spectrometry (LC-MS)). ^{1}H NMR and ^{13}C NMR were recorded on a Mercury300, Mercury400, Bruker AVANCE 400 spectrometer. High-resolution mass spectrometry (HRMS) data were measured on a Thermo Exactive Orbitrap plus spectrometer. LC-MS was conducted on an Agilent 1100 series HPLC and an Agilent LC/MSD TOF. Column chromatography was carried out using silica gel 60. UV−visible spectra were acquired with a Shimadzu UV-2700, UV−vis spectrophotometer. Fluorescence properties were measured with a HITACHI F-7000 fluorescence spectrophotometer. Fluorescence lifetime was measured with Edinburgh Analytical Instruments F900. The purity of all final compounds was 95% or higher. LC-MS was performed on a Thermo Fisher Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Thermo Fisher Accela HPLC system (Thermo Fisher Scientific, Bremen, Germany). Chromatographic separation was achieved on a Zorbax SB-C18 column (50 mm × 2.1 mm, 5 μm particle size) connected to an Agilent guard column. A linear gradient from 95% A (0.1% formic acid in water) to 95% B (0.1% formic acid in acetonitrile) in 5 min at a flow rate of 0.3 mL/min and detection wavelength of 254 nm were applied. The mass spectrometric data was collected from m/z 100 to 1500 in a positive ion mode. Nitrogen was used as the sheath and auxiliary gas with a flow rate of 40 psi and 10 L/min, respectively. The data was acquired in full scan MS at a resolving power of 17 500. The other mass parameters were as follows: the capillary temperature at 350 °C, ion spray voltage at 3.0 kV, heater temp at 300 °C, automatic gain control (AGC) target at 3×10^6, maximum IT at 50 ms.

General Experimental Procedure for 7-Hydroxychromone Analogues (I1 to VI1). The mixture of phenol derivatives (1 equiv, 3 mmol), acetic acid derivatives (1 equiv, 3 mmol), and $BF_3{\cdot}Et_2O$ (5 equiv, 15 mmol) was heated to 90 °C under N_2. The reaction was analyzed by thin-layer chromatography (TLC). After the completion of the reaction, the reaction mixture was cooled to 10 °C in an ice bath, and dried DMF (20 equiv, 60 mmol) was added dropwise. In another flask, 2,4,6-trichloro-1,3,5-triazine (TCT) (1.5 equiv, 4.5 mmol) was added into anhydrous DMF (10 equiv, 30 mmol) and stirred at room temperature (r.t.) for 15 min (TCT disappearance was monitored by thin-layer chromatography). The above reaction mixture was then added dropwise to the white suspension containing the TCT/DMF adduct at room temperature for 5 min. After the

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formation of a clear solution, the reaction mixture was heated to 60 °C. The reaction was monitored by TLC and completed after 3−5 h. Then, the reaction mixture was poured into boiling dilute HCl (20 mL) slowly and cooled to room temperature. The solution was extracted with ethyl acetate (30 mL × 3). The organic phase was washed with brine, dried, and concentrated in vacuo, which was purified by silica gel column (polyethylene (PE)/ethyl acetate (EA) = 100:20).

7-Hydroxy-3-(p-tolyl)-4H-chromen-4-one (I1). Compound (I1) was prepared according to the general procedure and the product was obtained as a pale yellow solid in 88.5% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.76 (s, 1H), 8.31 (s, 1H), 7.93 (d, J = 8.8 Hz, 1H), 7.48−7.38 (m, 2H), 7.26−7.15 (m, 2H), 6.90 (dd, J = 8.8, 2.2 Hz, 1H), 6.83 (d, J = 2.2 Hz, 1H), 2.30 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.94, 163.05, 157.89, 153.94, 137.47, 129.21, 129.14, 127.77, 123.89, 117.09, 115.67, 102.61, 21.27. HRMS (electrospray ionization (ESI)): m/z calcd for $C_{16}H_{13}O_3$ [M + H]$^+$, 253.0856; found, 253.0859.

7-Hydroxy-3-methyl-4H-chromen-4-one (II1). Compound (II1) was prepared according to the general procedure and the product was obtained as a white solid in 81.6% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.65 (s, 1H), 8.06 (s, 1H), 7.84 (d, J = 8.7 Hz, 1H), 6.85 (dd, J = 8.7, 2.1 Hz, 1H), 6.76 (d, J = 2.0 Hz, 1H), 1.82 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.75, 162.75, 158.36, 152.55, 127.13, 119.62, 116.35, 115.33, 102.53, 11.16. HRMS (ESI): m/z calcd for $C_{10}H_9O_3$ [M + H]$^+$, 177.0542; found, 177.0546.

7-Hydroxy-3-propyl-4H-chromen-4-one (III1). Compound (III1) was prepared according to the general procedure and the product was obtained as a white solid in 83.3% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.64 (s, 1H), 8.02 (s, 1H), 7.87−7.80 (m, 1H), 6.84 (dd, J = 8.7, 2.3 Hz, 1H), 6.75 (d, J = 2.2 Hz, 1H), 2.29−2.20 (m, 2H), 1.46 (dt, J = 14.8, 7.4 Hz, 2H), 0.83 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.31, 162.76, 158.19, 152.95, 127.24, 123.33, 116.61, 115.32, 102.52, 27.47, 21.73, 14.11. HRMS (ESI): m/z calcd for $C_{12}H_{13}O_3$ [M + H]$^+$, 205.0858; found, 205.0859.

7-Hydroxy-6-methoxy-3-(p-tolyl)-4H-chromen-4-one (IV1). Compound (IV1) was prepared according to the general procedure and the product was obtained as a white solid in 69.9% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.56 (s, 1H), 8.30 (s, 1H), 7.46−7.38 (m, 3H), 7.19 (d, J = 8.0 Hz, 2H), 6.92 (s, 1H), 3.84 (s, 3H), 2.30 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.65, 153.66, 153.47, 152.26, 147.51, 137.47, 129.92, 129.22, 123.44, 116.80, 105.26, 103.40, 56.36, 21.34. HRMS (ESI): m/z calcd for $C_{17}H_{15}O_4$ [M + H]$^+$, 283.0956; found, 283.0965.

7-Hydroxy-6-methoxy-3-methyl-4H-chromen-4-one (V1). Compound (V1) was prepared according to the general procedure and the product was obtained as a white solid in 73.1% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.45 (s, 1H), 8.09−8.03 (m, 1H), 7.31 (s, 1H), 6.84 (s, 1H), 3.82 (s, 3H), 1.88−1.80 (m, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.52, 153.19, 152.34, 147.24, 119.14, 115.99, 104.68, 103.37, 56.33, 11.36. HRMS (ESI): m/z calcd for $C_{11}H_{11}O_4$ [M + H]$^+$, 207.0646; found, 207.0652.

7-Hydroxy-6-methoxy-3-propyl-4H-chromen-4-one (VI1). Compound (VI1) was prepared according to the general procedure and the product was obtained as a white solid in 71.3% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.44 (s, 1H), 8.01 (s, 1H), 7.31 (s, 1H), 6.84 (s, 1H), 3.82 (s, 3H), 2.30−2.22 (m, 2H), 1.46 (h, J = 7.4 Hz, 2H), 0.83 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.06, 153.19, 152.56, 147.21, 122.86, 116.25, 104.80, 103.34, 56.29, 27.65, 21.81, 14.15. HRMS (ESI): m/z calcd for $C_{13}H_{15}O_4$ [M + H]$^+$, 235.0959; found, 235.0965.

General Experimental Procedure for 7-Triflate-Substituted Chromone Analogues (I7, II7, III9, IV8, V8, and VI9). To a cooled (0 °C) biphasic mixture of toluene (10 mL), 30% (w/v) aqueous K_3PO_4 (10 mL), and the 7-hydroxychromone analogue (1 equiv, 2 mmol) was added triflic anhydride (Tf_2O) (1.5 equiv, 3 mmol) dropwise at a rate to maintain the reaction temperature <10 °C. The reaction mixture was allowed to warm to ambient temperature. The reaction was monitored by TLC and completed after 5−8 h. The phases were separated, and the aqueous phase was extracted with ethyl acetate (20 mL × 3). The organic phase was washed with brine, dried, and concentrated in vacuo, which was purified by silica gel column (PE/EA = 100:5).

4-Oxo-3-(p-tolyl)-4H-chromen-7-yl Trifluoromethanesulfonate (I7). Compound (I7) was prepared according to the general procedure and the product was obtained as a yellowish-brown solid in 87.3% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.56 (s, 1H), 8.27 (d, J = 8.8 Hz, 1H), 8.07 (d, J = 2.3 Hz, 1H), 7.61 (dd, J = 8.9, 2.4 Hz, 1H), 7.49−7.41 (m, 2H), 7.26−7.19 (m, 2H), 2.31 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.81, 156.34, 155.49, 152.17, 138.01, 129.27, 129.22, 129.03, 128.77, 124.79, 124.37, 119.45, 112.94, 21.30. HRMS (ESI): m/z calcd for $C_{17}H_{12}O_5F_3S$ [M + H]+, 385.0343; found, 385.0352.

3-Methyl-4-oxo-4H-chromen-7-yl Trifluoromethanesulfonate (II7). Compound (II7) was prepared according to the general procedure and the product was obtained as a yellowish solid in 83.9% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.18 (s, 1H), 8.08 (t, J = 10.6 Hz, 1H), 7.76 (s, 1H), 7.43 (t, J = 11.3 Hz, 1H), 1.81 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.28, 156.61, 153.88, 151.85, 128.27, 123.16, 120.90, 118.79, 112.43, 10.83. HRMS (ESI): m/z calcd for $C_{11}H_8O_5F_3S$ [M + H]$^+$, 309.0031; found, 309.0039.

4-Oxo-3-propyl-4H-chromen-7-yl Trifluoromethanesulfonate (III9). Compound (III9) was prepared according to the general procedure and the product was obtained as a yellowish-brown solid in 89.2% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26 (s, 1H), 8.17 (d, J = 8.9 Hz, 1H), 7.94 (t, J = 2.5 Hz, 1H), 7.53 (dd, J = 8.9, 2.3 Hz, 1H), 2.30 (t, J = 7.5 Hz, 2H), 1.48 (h, J = 7.4 Hz, 2H), 0.85 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.08, 156.61, 154.57, 151.98, 128.51, 124.57, 123.61, 119.07, 112.77, 27.34, 21.46, 13.99. HRMS (ESI): m/z calcd for $C_{13}H_{12}O_5F_3S$ [M + H]$^+$, 337.0344; found, 337.0352.

6-Methoxy-4-oxo-3-(p-tolyl)-4H-chromen-7-yl Trifluoromethanesulfonate (IV8). Compound (IV8) was prepared according to the general procedure and the product was obtained as a yellowish-brown solid in 78.9% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.54 (s, 1H), 8.09 (s, 1H), 7.76 (s, 1H), 7.45 (d, J = 8.1 Hz, 2H), 7.22 (d, J = 8.2 Hz, 2H), 3.99 (s, 3H), 2.31 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.50, 155.42, 149.76, 149.07, 141.90, 137.91, 129.01, 124.86, 123.96, 114.41, 108.13, 57.52, 21.29. HRMS (ESI): m/z calcd for $C_{18}H_{14}O_6F_3S$ [M + H]$^+$, 415.0454; found, 415.0458.

6-Methoxy-3-methyl-4-oxo-4H-chromen-7-yl Trifluoromethanesulfonate (V8). Compound (V8) was prepared according to the general procedure and the product was obtained as a yellowish-brown solid in 78.6% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.30 (t, J = 1.1 Hz, 1H), 7.98 (s, 1H), 7.67 (s, 1H), 3.96 (s, 3H), 1.89 (d, J = 1.1 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.29, 154.14, 150.20, 148.80, 141.76, 123.81, 120.05, 114.30, 107.50, 57.47, 11.20. HRMS (ESI): m/z calcd for $C_{12}H_{10}O_6F_3S$ [M + H]$^+$, 339.0137; found, 339.0145.

6-Methoxy-4-oxo-3-propyl-4H-chromen-7-yl Trifluoromethanesulfonate (VI9). Compound (VI9) was prepared according to the general procedure and the product was obtained as a yellowish-brown solid in 71.3% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.21 (s, 1H), 7.90 (s, 1H), 7.64 (s, 1H), 3.94 (s, 3H), 2.33−2.25 (m, 2H), 1.47 (h, J = 7.4 Hz, 2H), 0.84 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 175.84, 154.46, 150.07, 148.83, 141.81, 124.11, 123.75, 114.20, 107.63, 57.41, 27.49, 21.54, 13.99. HRMS (ESI): m/z calcd for $C_{14}H_{14}O_6F_3S$ [M + H]$^+$, 367.0442; found, 367.0458.

General Experimental Procedure for 7-Amino Chromone Analogues (I2, II2, III2, IV2, V2, and VI2). To a mixture of palladium acetate (0.03 equiv, 3 mol %), BINAP (0.05 equiv, 5 mol %), and cesium carbonate (3 equiv, 3 mmol) were added triflate (1 equiv, 1 mmol) and amine (1.5 equiv, 1.5 mmol) in toluene. The mixture was stirred for 30 min at room temperature, heated to 80 °C under N_2, and stirred overnight at the same temperature. The reaction mixture was cooled to room temperature, filtered through the Celite pad, and concentrated in vacuo. The residue was dissolved in the mixture solvent THF (10 mL) and 2 N aqueous HCl (10 mL) and stirred at room temperature. The reaction was monitored by TLC and completed after 2−5 h. Then, 2 N aqueous NaOH was added to

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adjust the pH to 10.0. The solution was extracted with ethyl acetate (30 mL × 3). The organic phase was washed with brine, dried, and concentrated in vacuo, which was purified by silica gel column (PE/EA = 100:30).

7-Amino-3-(p-tolyl)-4H-chromen-4-one (I2). Compound (I2) was prepared according to the general procedure and the product was obtained as a white solid in 49.3% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.20 (s, 1H), 7.78 (d, J = 8.8 Hz, 1H), 7.44 (d, J = 8.1 Hz, 2H), 7.22 (d, J = 7.9 Hz, 2H), 6.69 (dd, J = 8.7, 2.1 Hz, 1H), 6.51 (d, J = 2.1 Hz, 1H), 6.32 (s, 2H), 2.34 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.44, 158.43, 154.82, 152.96, 137.19, 130.05, 129.19, 127.18, 123.55, 113.98, 97.98, 21.27. HRMS (ESI): m/z calcd for $C_{16}H_{14}O_2N$ $[M + H]^+$, 252.1014; found, 252.1019.

7-Amino-3-methyl-4H-chromen-4-one (II2). Compound (II2) was prepared according to the general procedure and the product was obtained as a white solid in 55.3% yield. ^{1}H NMR (400 MHz, chloroform-d) δ 8.01 (d, J = 8.6 Hz, 1H), 7.63 (q, J = 1.2 Hz, 1H), 6.66 (dd, J = 8.6, 2.2 Hz, 1H), 6.51 (d, J = 2.2 Hz, 1H), 4.22 (s, 2H), 1.98 (d, J = 1.2 Hz, 3H). ^{13}C NMR (101 MHz, chloroform-d) δ 177.61, 158.74, 151.37, 150.73, 127.42, 120.10, 115.71, 113.59, 99.94, 11.23. HRMS (ESI): m/z calcd For $C_{10}H_{10}O_2N$ $[M + H]^+$, 176.0706; found, 176.0706.

7-Amino-3-propyl-4H-chromen-4-one (III2). Compound (III2) was prepared according to the general procedure and the product was obtained as a white solid in 45.2% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.89 (s, 1H), 7.71 (d, J = 8.7 Hz, 1H), 6.68−6.62 (m, 1H), 6.46 (d, J = 1.5 Hz, 1H), 6.22 (s, 2H), 2.26 (t, J = 7.5 Hz, 2H), 1.48 (h, J = 7.3 Hz, 2H), 0.87 (t, J = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 175.87, 158.74, 154.51, 151.87, 126.66, 122.87, 113.69, 113.55, 98.03, 27.57, 21.83, 14.11. HRMS (ESI): m/z calcd for $C_{12}H_{14}O_2N$ $[M + H]^+$, 204.1034; found, 204.1019.

7-Amino-6-methoxy-3-(p-tolyl)-4H-chromen-4-one (IV2). Compound (IV2) was prepared according to the general procedure and the product was obtained as a white solid in 46.7% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.22 (s, 1H), 7.46 (d, J = 8.1 Hz, 2H), 7.29 (s, 1H), 7.22 (d, J = 7.9 Hz, 2H), 6.65 (s, 1H), 6.08 (s, 2H), 3.89 (s, 3H), 2.34 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.16, 153.31, 152.58, 145.47, 145.38, 137.14, 130.27, 129.20, 129.08, 123.08, 113.38, 103.41, 98.29, 56.12, 21.27. HRMS (ESI): m/z calcd for $C_{17}H_{16}O_3N$ $[M + H]^+$, 282.1125; found, 282.1126.

7-Amino-6-methoxy-3-methyl-4H-chromen-4-one (V2). Compound (V2) was prepared according to the general procedure and the product was obtained as a white solid in 43.4% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.98 (s, 1H), 7.20 (s, 1H), 6.57 (s, 1H), 5.97 (s, 2H), 3.85 (s, 3H), 1.85 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.07, 153.79, 151.20, 145.24, 145.07, 118.62, 112.72, 102.86, 98.38, 56.08, 11.39. HRMS (ESI): m/z calcd for $C_{11}H_{12}O_3N$ $[M + H]^+$, 206.0812; found, 206.0812.

7-Amino-6-methoxy-3-propyl-4H-chromen-4-one (VI2). Compound (VI2) was prepared according to the general procedure and the product was obtained as a white solid in 43.8% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.92 (s, 1H), 7.21 (s, 1H), 6.59 (s, 1H), 5.98 (s, 2H), 3.86 (s, 3H), 2.35−2.24 (m, 2H), 1.50 (dt, J = 14.8, 7.4 Hz, 2H), 0.87 (t, J = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 175.62, 153.61, 151.52, 145.22, 145.06, 122.39, 112.99, 102.98, 98.37, 56.03, 27.68, 21.84, 14.11. HRMS (ESI): m/z calcd for $C_{13}H_{16}O_3N$ $[M + H]^+$, 234.1120; found, 234.1125.

General Experimental Procedure for 7-Ethylamino Chromone Analogues (III3, V3, and VI3). 25 mL flask equipped with a stir bar was charged with 7-amino chromone analogues (1 equiv, 1 mmol) and KOH (1.5 equiv, 1.5 mmol). Then, 10 mL of DMSO was added to the flask and the solution was stirred at room temperature, followed by the addition of bromoethane (1 equiv, 1 mmol). The reaction mixture was stirred at 50 °C overnight. The reaction mixture was quenched with water (10 mL) and extracted with ethyl acetate (3 × 20 mL). The organic phase was washed with brine, dried, and concentrated in vacuo, which was purified by silica gel column (PE/EA = 100:15).

7-(Ethylamino)-3-propyl-4H-chromen-4-one (III3). Compound (III3) was prepared according to the general procedure and the product was obtained as a white solid in 18.6% yield. ^{1}H NMR (400 MHz, chloroform-d) δ 7.95 (d, J = 8.8 Hz, 1H), 7.56 (s, 1H), 6.57 (dd, J = 8.8, 2.2 Hz, 1H), 6.34 (d, J = 2.2 Hz, 1H), 3.21 (q, J = 7.2 Hz, 2H), 2.42−2.33 (m, 2H), 1.58 (h, J = 7.4 Hz, 2H), 1.28 (t, J = 7.2 Hz, 3H), 0.93 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, chloroform-d) δ 152.45, 150.83, 126.90, 123.81, 112.82, 96.22, 38.11, 27.83, 21.70, 14.41, 13.87. HRMS (ESI): m/z calcd for $C_{14}H_{18}O_2N$ $[M + H]^+$, 232.1332; found, 232.1336.

7-(Ethylamino)-6-methoxy-3-methyl-4H-chromen-4-one (V3). Compound (V3) was prepared according to the general procedure and the product was obtained as a white solid in 20.4% yield. ^{1}H NMR (400 MHz, chloroform-d) δ 7.63 (s, 1H), 7.39 (s, 1H), 6.35 (s, 1H), 3.93 (s, 3H), 3.22 (q, J = 7.2 Hz, 2H), 2.00 (d, J = 1.2 Hz, 3H), 1.32 (t, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, chloroform-d) δ 154.54, 150.17, 144.86, 143.80, 119.44, 113.01, 102.13, 94.87, 55.96, 37.74, 14.28, 11.38. HRMS (ESI): m/z calcd for $C_{13}H_{16}O_3N$ $[M + H]^+$, 234.1125; found, 234.1142.

7-(Ethylamino)-6-methoxy-3-propyl-4H-chromen-4-one (VI3). Compound (VI3) was prepared according to the general procedure and the product was obtained as a white solid in 19.0% yield. ^{1}H NMR (400 MHz, chloroform-d) δ 7.64 (s, 1H), 7.51 (s, 1H), 6.76 (s, 1H), 4.22−4.06 (m, 2H), 3.92 (s, 3H), 2.45−2.33 (m, 2H), 1.63−1.52 (m, 2H), 1.50 (t, J = 7.0 Hz, 3H), 0.93 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, chloroform-d) δ 176.99, 153.48, 152.55, 151.28, 147.51, 123.74, 117.12, 104.44, 100.08, 64.88, 56.27, 27.86, 21.62, 14.44, 13.84. HRMS (ESI): m/z calcd for $C_{15}H_{20}O_3N$ $[M + H]^+$, 262.1438; found, 262.1438.

General Experimental Procedure for Amination of Triflates (I3−6, II3−6, III4−8, IV3−7, V4−7, and VI4−8). To a mixture of palladium acetate (0.03 equiv, 3 mol %), BINAP (0.05 equiv, 5 mol %), and cesium carbonate (3 equiv, 3 mmol) were added triflate (1 equiv, 1 mmol) and amine (1.5 equiv, 1.5 mmol) in toluene. The mixture was stirred for 30 min at room temperature, heated to 80 °C under N_2, and stirred overnight at the same temperature. The reaction mixture was cooled to room temperature and filtered through the Celite pad. The residue was purified by flash column chromatography on silica gel to afford the corresponding coupling product.

7-(Diethylamino)-3-(p-tolyl)-4H-chromen-4-one (I3). Compound (I3) was prepared according to the general procedure and the product was obtained as a white solid in 38.9% yield. ^{1}H NMR (400 MHz, chloroform-d) δ 8.08 (d, J = 9.1 Hz, 1H), 7.80 (d, J = 3.3 Hz, 1H), 7.45 (d, J = 8.0 Hz, 2H), 7.25−7.18 (m, 2H), 6.73 (dd, J = 9.1, 2.4 Hz, 1H), 6.43 (d, J = 2.3 Hz, 1H), 3.43 (q, J = 7.1 Hz, 4H), 2.37 (s, 3H), 1.22 (t, J = 7.1 Hz, 6H); ^{13}C NMR (100 MHz, chloroform-d) δ 175.46, 158.72, 151.60, 137.53, 129.66, 129.05, 128.88, 127.55, 124.67, 113.84, 110.61, 96.20, 44.78, 21.28, 12.49. HRMS (ESI): m/z calcd for $C_{20}H_{22}O_2N$ $[M + H]^+$, 308.1641; found, 308.1645.

7-(Pyrrolidin-1-yl)-3-(p-tolyl)-4H-chromen-4-one (I4). Compound (I4) was prepared according to the general procedure and the product was obtained as a white solid in 63.1% yield. ^{1}H NMR (400 MHz, chloroform-d) δ 8.16−8.06 (m, 1H), 7.86−7.77 (m, 1H), 7.45 (d, J = 8.1 Hz, 2H), 7.23−7.18 (m, 2H), 6.64 (dd, J = 8.9, 2.2 Hz, 1H), 6.38−6.31 (m, 1H), 3.39 (t, J = 6.6 Hz, 4H), 2.37 (s, 3H), 2.05−2.08 (m, 4H); ^{13}C NMR (100 MHz, chloroform-d) δ 175.75, 158.44, 151.49, 137.57, 129.61, 129.45, 129.22, 129.07, 128.91, 127.45, 124.67, 113.97, 111.34, 96.50, 47.81, 31.96, 29.40, 25.48, 22.73, 14.16. HRMS (ESI): m/z calcd for $C_{20}H_{20}O_2N$ $[M + H]^+$, 306.1484; found, 306.1489.

7-(Piperidin-1-yl)-3-(p-tolyl)-4H-chromen-4-one (I5). Compound (I5) was prepared according to the general procedure and the product was obtained as a white solid in 54.9% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.25 (s, 1H), 7.84 (d, J = 9.0 Hz, 1H), 7.42 (d, J = 8.0 Hz, 2H), 7.18 (d, J = 7.8 Hz, 2H), 6.83 (s, 1H), 3.40 (d, J = 5.1 Hz, 4H), 2.30 (s, 3H), 1.57 (m, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.55, 158.36, 154.89, 153.51, 137.29, 129.89, 129.10, 126.88, 123.76, 114.55, 113.59, 99.50, 48.32, 25.27, 24.40, 21.28. HRMS (ESI): m/z calcd for $C_{21}H_{22}O_2N$ $[M + H]^+$, 320.1638; found, 320.1645.

7-Morpholino-3-(p-tolyl)-4H-chromen-4-one (I6). Compound (I6) was prepared according to the general procedure and the

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product was obtained as a white solid in 59.1% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 8.13 (d, *J* = 9.0 Hz, 1H), 7.87 (s, 1H), 7.43 (d, *J* = 8.1 Hz, 2H), 7.22 (d, *J* = 8.0 Hz, 2H), 6.96 (dd, *J* = 9.0, 2.3 Hz, 1H), 6.70 (d, *J* = 2.2 Hz, 1H), 3.92−3.83 (m, 4H), 3.36−3.29 (m, 4H), 2.36 (s, 3H). ^{13}C NMR (100 MHz, chloroform-*d*) δ 175.77, 158.16, 154.92, 152.19, 137.83, 129.14, 128.85, 127.44, 125.07, 113.08, 100.15, 66.50, 47.64, 21.27. HRMS (ESI): *m*/*z* calcd for $C_{20}H_{20}O_3N$ [M + H]$^+$, 322.1433; found, 322.1438.

7-(Diethylamino)-3-methyl-4H-chromen-4-one (II3). Compound (II3) was prepared according to the general procedure and the product was obtained as a white solid in 28.1% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.99 (d, *J* = 9.1 Hz, 1H), 7.58 (q, *J* = 1.2 Hz, 1H), 6.69 (dd, *J* = 9.1, 2.5 Hz, 1H), 6.38 (d, *J* = 2.5 Hz, 1H), 3.40 (q, *J* = 7.1 Hz, 4H), 1.96 (d, *J* = 1.2 Hz, 3H), 1.20 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, chloroform-*d*) δ 177.51, 159.18, 150.43, 126.95, 119.81, 110.39, 96.18, 44.73, 29.72, 12.47, 11.26. HRMS (ESI): *m*/*z* calcd for $C_{14}H_{18}O_2N$ [M + H]$^+$, 232.1328; found, 232.1332.

3-Methyl-7-(pyrrolidin-1-yl)-4H-chromen-4-one (II4). Compound (II4) was prepared according to the general procedure and the product was obtained as a white solid in 59.3% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.96 (s, 1H), 7.76 (d, *J* = 8.9 Hz, 1H), 6.65 (dd, *J* = 8.9, 2.2 Hz, 1H), 6.33 (d, *J* = 2.1 Hz, 1H), 3.30−3.27 (m, 4H), 1.95−1.91 (m, 4H), 1.80 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.43, 158.68, 151.72, 151.47, 126.41, 119.23, 111.70, 96.76, 47.91, 25.40, 11.27. HRMS (ESI): *m*/*z* calcd for $C_{14}H_{16}O_2N$ [M + H]$^+$, 230.1173; found, 230.1173.

3-Methyl-7-(piperidin-1-yl)-4H-chromen-4-one (II5). Compound (II5) was prepared according to the general procedure and the product was obtained as a white solid in 53.2% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 8.00 (d, *J* = 9.1 Hz, 1H), 7.61 (q, *J* = 1.2 Hz, 1H), 6.91 (dd, *J* = 9.1, 2.4 Hz, 1H), 6.61 (d, *J* = 2.3 Hz, 1H), 3.34 (t, *J* = 5.2 Hz, 4H), 1.96 (d, *J* = 1.2 Hz, 3H), 1.71−1.60 (m, 6H). ^{13}C NMR (101 MHz, chloroform-*d*) δ 154.80, 150.77, 126.62, 120.08, 113.25, 99.77, 48.83, 25.30, 24.30, 11.24. HRMS (ESI): *m*/*z* calcd for $C_{15}H_{18}O_2N$ [M + H]$^+$, 244.1329; found, 244.1332.

3-Methyl-7-morpholino-4H-chromen-4-one (II6). Compound (II6) was prepared according to the general procedure and the product was obtained as a white solid in 61.2% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.04 (s, 1H), 7.80 (d, *J* = 8.9 Hz, 1H), 7.06 (dd, *J* = 9.1, 2.3 Hz, 1H), 6.81 (d, *J* = 2.1 Hz, 1H), 3.70 (t, *J* = 4.7 Hz, 4H), 3.28 (t, *J* = 4.9 Hz, 4H), 1.82 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.54, 158.52, 154.99, 152.35, 126.23, 119.60, 114.96, 113.17, 100.10, 66.25, 47.33, 11.22. HRMS (ESI): *m*/*z* calcd for $C_{14}H_{16}O_3N$ [M + H]$^+$, 246.1119; found, 246.1125.

7-(Diethylamino)-3-propyl-4H-chromen-4-one (III4). Compound (III4) was prepared according to the general procedure and the product was obtained as a white solid in 39.8% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 8.00 (d, *J* = 9.1 Hz, 1H), 7.54 (t, *J* = 0.8 Hz, 1H), 6.70 (dd, *J* = 9.1, 2.5 Hz, 1H), 6.40 (s, 1H), 3.41 (q, *J* = 7.1 Hz, 4H), 2.41−2.32 (m, 2H), 1.63−1.53 (m, 2H), 1.20 (t, *J* = 7.1 Hz, 6H), 0.94 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, chloroform-*d*) δ 177.04, 158.95, 150.72, 129.05, 128.24, 127.06, 123.64, 110.40, 96.29, 44.79, 29.72, 27.84, 21.74, 13.87, 12.46. HRMS (ESI): *m*/*z* calcd for $C_{16}H_{22}O_2N$ [M + H]$^+$, 260.1643; found, 260.1645.

7-(Azetidin-1-yl)-3-propyl-4H-chromen-4-one (III5). Compound (III5) was prepared according to the general procedure and the product was obtained as a white solid in 43.4% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 8.01 (d, *J* = 8.8 Hz, 1H), 7.56 (s, 1H), 6.41 (dd, *J* = 8.8, 2.2 Hz, 1H), 6.16−6.12 (m, 1H), 4.03−3.97 (m, 4H), 2.47−2.36 (m, 4H), 1.59 (h, *J* = 7.4 Hz, 2H), 0.94 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, chloroform-*d*) δ 150.93, 127.10, 123.85, 114.69, 109.63, 95.79, 77.16, 51.78, 27.96, 21.82, 16.64, 13.99. HRMS (ESI): *m*/*z* calcd for $C_{15}H_{18}O_2N$ [M + H]$^+$, 244.1332; found, 244.1323.

3-Propyl-7-(pyrrolidin-1-yl)-4H-chromen-4-one (III6). Compound (III6) was prepared according to the general procedure and the product was obtained as a white solid in 54.6% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 8.04 (d, *J* = 8.9 Hz, 1H), 7.56 (t, *J* = 0.9 Hz, 1H), 6.61 (dd, *J* = 8.9, 2.2 Hz, 1H), 6.29 (d, *J* = 2.2 Hz, 1H), 3.41−3.32 (m, 4H), 2.39 (td, *J* = 7.5, 0.8 Hz, 2H), 2.09−2.01 (m, 4H), 1.65−1.54 (m, 2H), 0.95 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, chloroform-*d*) δ 177.23, 158.66, 151.28, 150.71, 126.94, 123.60, 113.65, 110.97, 96.45, 47.75, 29.73, 27.87, 25.48, 21.73, 13.89. HRMS (ESI): *m*/*z* calcd for $C_{16}H_{20}O_2N$ [M + H]$^+$, 258.1486; found, 258.1489.

7-(Piperidin-1-yl)-3-propyl-4H-chromen-4-one (III7). Compound (III7) was prepared according to the general procedure and the product was obtained as a white solid in 53.8% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.99 (d, *J* = 9.1 Hz, 1H), 7.56 (s, 1H), 6.90 (dd, *J* = 9.1, 2.3 Hz, 1H), 6.60 (d, *J* = 2.1 Hz, 1H), 3.32 (d, *J* = 5.4 Hz, 4H), 2.41−2.33 (m, 2H), 1.65 (m, 6H), 1.56 (dt, *J* = 14.8, 7.5 Hz, 2H), 0.93 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, chloroform-*d*) δ 177.12, 158.64, 154.83, 151.04, 126.69, 123.85, 113.20, 99.71, 48.81, 27.83, 25.30, 24.31, 21.69, 13.87. HRMS (ESI): *m*/*z* calcd for $C_{17}H_{22}O_2N$ [M + H]$^+$, 272.1642; found, 272.1645.

7-Morpholino-3-propyl-4H-chromen-4-one (III8). Compound (III8) was prepared according to the general procedure and the product was obtained as a white solid in 56.7% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.00 (s, 1H), 7.80 (d, *J* = 9.1 Hz, 1H), 7.06 (dd, *J* = 9.1, 2.3 Hz, 1H), 6.81 (d, *J* = 2.3 Hz, 1H), 3.32−3.24 (m, 4H), 2.29−2.20 (m, 2H), 1.46 (h, *J* = 7.4 Hz, 2H), 0.84 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.10, 158.35, 155.02, 152.73, 126.33, 123.31, 115.22, 113.17, 100.11, 66.24, 47.36, 27.52, 21.74, 14.12. HRMS (ESI): *m*/*z* calcd for $C_{16}H_{20}O_3N$ [M + H]$^+$, 274.1429; found, 274.1438.

7-(Diethylamino)-6-methoxy-3-(p-tolyl)-4H-chromen-4-one (IV3). Compound (IV3) was prepared according to the general procedure and the product was obtained as a white solid in 25.9% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.89 (s, 1H), 7.57 (s, 1H), 7.46 (d, *J* = 8.1 Hz, 2H), 7.24 (d, *J* = 8.3 Hz, 2H), 6.78 (s, 1H), 3.95 (s, 3H), 3.36 (q, *J* = 7.0 Hz, 4H), 2.38 (s, 3H), 1.16 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (101 MHz, chloroform-*d*) δ 175.45, 152.49, 151.82, 150.35, 146.17, 137.65, 129.11, 128.87, 124.36, 117.31, 105.67, 105.05, 56.00, 45.81, 29.73, 21.28, 12.45. HRMS (ESI): *m*/*z* calcd for $C_{21}H_{24}O_3N$ [M + H]$^+$, 338.1751; found, 338.1746.

7-(Azetidin-1-yl)-6-methoxy-3-(p-tolyl)-4H-chromen-4-one (IV4). Compound (IV4) was prepared according to the general procedure and the product was obtained as a white solid in 32.7% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.84 (s, 1H), 7.47 (s, 1H), 7.46 (d, *J* = 3.1 Hz, 2H), 7.23 (d, *J* = 7.8 Hz, 2H), 6.20 (s, 1H), 4.18−4.10 (m, 4H), 3.87 (s, 3H), 2.43−2.31 (m, 5H). ^{13}C NMR (101 MHz, chloroform-*d*) δ 175.23, 153.24, 151.35, 147.57, 146.59, 137.53, 129.74, 129.07, 128.88, 124.23, 115.46, 104.40, 98.02, 55.83, 54.10, 21.27, 17.44. HRMS (ESI): *m*/*z* calcd for $C_{20}H_{20}O_3N$ [M + H]$^+$, 322.14377; found, 322.14435

6-Methoxy-7-(pyrrolidin-1-yl)-3-(p-tolyl)-4H-chromen-4-one (IV5). Compound (IV5) was prepared according to the general procedure and the product was obtained as a white solid in 41.7% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.82 (s, 1H), 7.49 (s, 1H), 7.46 (d, *J* = 8.0 Hz, 2H), 7.22 (d, *J* = 8.0 Hz, 2H), 6.44 (s, 1H), 3.88 (s, 3H), 3.52 (t, *J* = 6.5 Hz, 4H), 2.37 (s, 3H), 1.95 (t, *J* = 4.5 Hz, 4H); ^{13}C NMR (100 MHz, chloroform-*d*) δ 175.14, 153.37, 151.39, 147.81, 145.05, 137.48, 129.83, 129.06, 128.89, 124.20, 114.81, 104.98, 99.87, 56.00, 50.81, 25.50, 21.28. HRMS (ESI): *m*/*z* calcd for $C_{21}H_{22}O_3N$ [M + H]$^+$, 336.1585; found, 336.1594.

6-Methoxy-7-(piperidin-1-yl)-3-(p-tolyl)-4H-chromen-4-one (IV6). Compound (IV6) was prepared according to the general procedure and the product was obtained as a white solid in 43.1% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.90 (d, *J* = 1.0 Hz, 1H), 7.57 (s, 1H), 7.45 (d, *J* = 8.0 Hz, 2H), 7.22 (d, *J* = 8.0 Hz, 2H), 6.85 (s, 1H), 3.95 (s, 3H), 3.17−3.09 (m, 4H), 2.37 (s, 3H), 1.77 (p, *J* = 5.8 Hz, 4H), 1.62 (p, *J* = 5.8 Hz, 2H); ^{13}C NMR (100 MHz, chloroform-*d*) δ 175.57, 152.35, 152.01, 150.45, 148.75, 137.70, 129.48, 129.13, 128.86, 124.44, 118.48, 106.01, 104.84, 56.06, 51.76, 25.99, 24.30, 21.29. HRMS (ESI): *m*/*z* calcd for $C_{22}H_{24}O_3N$ [M + H]$^+$, 350.1744; found, 350.1751.

6-Methoxy-7-morpholino-3-(p-tolyl)-4H-chromen-4-one (IV7). Compound (IV7) was prepared according to the general procedure and the product was obtained as a white solid in 47.3% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.91 (s, 1H), 7.60 (s, 1H), 7.45 (d,

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J = 8.0 Hz, 2H), 7.24 (d, J = 3.4 Hz, 2H), 6.85 (s, 1H), 3.96 (s, 3H), 3.92−3.88 (m, 4H), 3.24−3.17 (m, 4H), 2.37 (s, 3H); ^{13}C NMR (100 MHz, chloroform-d) δ 175.52, 152.20, 152.14, 150.25, 147.22, 137.82, 129.30, 129.16, 128.84, 124.57, 119.05, 105.89, 105.25, 66.86, 56.08, 50.69, 21.29. HRMS (ESI): m/z calcd for $C_{21}H_{22}O_4N$ [M + H]$^+$, 352.1538; found, 352.1543.

7-(Diethylamino)-6-methoxy-3-methyl-4H-chromen-4-one (V4). Compound (V4) was prepared according to the general procedure and the product was obtained as a white solid in 18.6% yield. ^{1}H NMR (400 MHz, chloroform-d) δ 7.68 (s, 1H), 7.48 (s, 1H), 6.73 (s, 1H), 3.93 (s, 3H), 3.32 (q, J = 7.0 Hz, 4H), 2.01 (s, 3H), 1.13 (t, J = 7.0 Hz, 6H). HRMS (ESI): m/z calcd for $C_{15}H_{20}O_3N$ [M + H]$^+$, 262.1438; found, 262.1455.

6-Methoxy-3-methyl-7-(pyrrolidin-1-yl)-4H-chromen-4-one (V5). Compound (V5) was prepared according to the general procedure and the product was obtained as a white solid in 46.2% yield. ^{1}H NMR (400 MHz, chloroform-d) δ 7.68−7.61 (m, 1H), 7.50−7.44 (m, 1H), 6.76−6.71 (m, 1H), 3.91−3.86 (m, 3H), 3.85−3.81 (m, 4H), 3.16−3.10 (m, 4H), 1.96−1.93 (m, 3H); ^{13}C NMR (100 MHz, chloroform-d) δ 177.22, 153.83, 150.25, 147.65, 144.81, 119.42, 114.17, 104.43, 100.05, 55.98, 50.81, 25.47, 11.38. HRMS (ESI): m/z calcd for $C_{15}H_{18}O_3N$ [M + H]$^+$, 260.1278; found, 260.1281.

6-Methoxy-3-methyl-7-(piperidin-1-yl)-4H-chromen-4-one (V6). Compound (V6) was prepared according to the general procedure and the product was obtained as a white solid in 41.3% yield. ^{1}H NMR (400 MHz, chloroform-d) δ 7.65 (dd, J = 2.1, 1.1 Hz, 1H), 7.44 (d, J = 1.9 Hz, 1H), 6.76 (d, J = 2.2 Hz, 1H), 3.90 (d, J = 1.9 Hz, 3H), 3.10−3.03 (m, 4H), 1.96 (dd, J = 2.0, 1.2 Hz, 3H), 1.71 (p, J = 5.4, 5.0 Hz, 4H), 1.60−1.52 (m, 2H); ^{13}C NMR (100 MHz, chloroform-d) δ 177.56, 152.81, 150.88, 150.19, 148.53, 119.70, 117.58, 106.00, 104.20, 55.98, 51.72, 25.96, 24.26, 11.33. HRMS (ESI): m/z calcd for $C_{16}H_{20}O_3N$ [M + H]$^+$, 274.1530; found, 274.1438.

6-Methoxy-3-methyl-7-morpholino-4H-chromen-4-one (V7). Compound (V7) was prepared according to the general procedure and the product was obtained as a white solid in 49.7% yield. 1H NMR (400 MHz, chloroform-d) δ 7.68−7.61 (m, 1H), 7.50−7.44 (m, 1H), 6.76−6.71 (m, 1H), 3.91−3.86 (m, 3H), 3.85−3.81 (m, 4H), 3.16−3.10 (m, 4H), 1.96−1.93 (m, 3H); ^{13}C NMR (100 MHz, chloroform-d) δ 177.39, 152.64, 150.93, 149.98, 146.98, 119.85, 118.10, 105.78, 104.60, 66.78, 55.88, 50.63, 11.20. HRMS (ESI): m/z calcd for $C_{15}H_{18}O_4N$ [M + H]$^+$, 276.1221; found, 276.1230.

7-(Diethylamino)-6-methoxy-3-propyl-4H-chromen-4-one (VI4). Compound (VI4) was prepared according to the general procedure and the product was obtained as a white solid in 23.7% yield. ^{1}H NMR (400 MHz, chloroform-d) δ 7.63 (s, 1H), 7.47 (s, 1H), 6.72 (s, 1H), 3.92 (s, 3H), 3.31 (q, J = 7.0 Hz, 4H), 2.49−2.34 (m, 2H), 1.63−1.56 (m, 2H), 1.12 (t, J = 7.0 Hz, 6H), 0.94 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, chloroform-d) δ 177.03, 152.71, 150.97, 150.15, 145.94, 123.42, 116.89, 105.88, 104.53, 55.94, 45.76, 27.92, 21.71, 13.86, 12.39. HRMS (ESI): m/z calcd for $C_{17}H_{24}O_3N$ [M + H]$^+$, 290.1751; found, 290.1756.

7-(Azetidin-1-yl)-6-methoxy-3-propyl-4H-chromen-4-one (VI5). Compound (VI5) was prepared according to the general procedure and the product was obtained as a white solid in 30.3% yield. ^{1}H NMR (400 MHz, chloroform-d) δ 7.61 (s, 1H), 7.42 (s, 1H), 6.43 (s, 1H), 3.93 (s, 3H), 3.67 (t, J = 6.2 Hz, 2H), 3.43 (t, J = 6.7 Hz, 2H), 2.41 (t, J = 7.5 Hz, 2H), 2.14 (p, J = 6.5 Hz, 2H), 1.60 (dt, J = 14.7, 7.4 Hz, 2H), 0.95 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, chloroform-d) δ 176.84, 154.16, 150.56, 144.90, 143.42, 123.29, 102.52, 95.09, 56.01, 42.33, 40.16, 31.43, 27.92, 21.71, 13.87. HRMS (ESI): m/z calcd for $C_{16}H_{20}O_3N$ [M + H]$^+$, 274.1438; found, 274.1436.

6-Methoxy-3-propyl-7-(pyrrolidin-1-yl)-4H-chromen-4-one (VI6). Compound (VI6) was prepared according to the general procedure and the product was obtained as a white solid in 49.2% yield. ^{1}H NMR (400 MHz, chloroform-d) δ 7.55 (s, 1H), 7.40 (s, 1H), 6.39 (s, 1H), 3.84 (s, 3H), 3.47 (t, J = 6.5 Hz, 4H), 2.42−2.34 (m, 2H), 1.58 (dq, J = 14.6, 7.4 Hz, 4H), 0.93 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, chloroform-d) δ 176.79, 153.62, 150.55, 147.63, 123.19, 104.51, 100.02, 55.93, 50.79, 27.94, 25.46, 21.72, 13.87. HRMS (ESI): m/z calcd for $C_{17}H_{22}O_3N$ [M + H]$^+$, 288.1586; found, 288.1594.

6-Methoxy-7-(piperidin-1-yl)-3-propyl-4H-chromen-4-one (VI7). Compound (VI7) was prepared according to the general procedure and the product was obtained as a white solid in 42.7% yield. ^{1}H NMR (400 MHz, chloroform-d) δ 7.63 (d, J = 0.8 Hz, 1H), 7.47 (s, 1H), 6.79 (s, 1H), 3.92 (s, 3H), 3.12−3.04 (m, 4H), 2.46−2.35 (m, 2H), 1.73 (p, J = 5.9 Hz, 4H), 1.63−1.49 (m, 4H), 0.92 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, chloroform-d) δ 177.20, 152.62, 151.22, 150.18, 148.55, 123.51, 117.91, 106.03, 104.32, 55.97, 51.77, 27.90, 25.96, 24.26, 21.67, 13.84. HRMS (ESI): m/z calcd for $C_{18}H_{24}O_3N$ [M + H]$^+$, 302.1735; found, 302.1751.

6-Methoxy-7-morpholino-3-propyl-4H-chromen-4-one (VI8). Compound (VI8) was prepared according to the general procedure and the product was obtained as a white solid in 41.9% yield. ^{1}H NMR (400 MHz, chloroform-d) δ 7.55 (s, 1H), 7.40 (s, 1H), 6.39 (s, 1H), 3.84 (s, 3H), 3.47 (t, J = 6.5 Hz, 4H), 2.42−2.34 (m, 2H), 1.58 (dq, J = 14.6, 7.4 Hz, 4H), 0.93 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, chloroform-d) δ 177.12, 152.45, 151.39, 149.97, 146.87, 123.69, 118.54, 106.03, 104.78, 66.82, 56.02, 50.71, 27.88, 21.64, 13.84. HRMS (ESI): m/z calcd for $C_{17}H_{22}O_4N$ [M + H]$^+$, 304.1536; found, 304.1543.

Synthesis of Various Chromone-Based Fluorescent Probes. *3-(4-Chlorobutyl)-7-hydroxy-6-methoxy-4H-chromen-4-one (VI10).* A mixture of 4-methoxybenzene-1,3-diol (1 equiv, 3 mmol), 6-chlorohexanoic acid (1 equiv, 3 mmol), and $BF_3{\cdot}Et_2O$ (5 equiv, 15 mmol) was heated to 90 °C under N_2. The reaction was analyzed by TLC for completion, and the reaction mixture was then cooled to 10 °C in an ice bath and dried DMF (20 equiv, 60 mmol) was added dropwise. In another flask, 2,4,6-trichloro-1,3,5-triazine (TCT) (1.5 equiv, 4.5 mmol) was added to anhydrous DMF (10 equiv, 30 mmol) and stirred at room temperature for 15 min (TCT disappearance was monitored by thin-layer chromatography). The above reaction mixture was then added dropwise to the white suspension containing the TCT/DMF adduct at room temperature for 5 min. After the formation of a clear solution, the reaction mixture was heated to 60 °C. The reaction was monitored by TLC and completed after 3−5 h. Then, the reaction mixture was poured into boiling dilute HCl (20 mL) slowly and cooled to room temperature. The solution was extracted with ethyl acetate (30 mL × 3). The organic phase was washed with brine, dried, and concentrated in vacuo, which was purified by silica gel column (PE/EA = 100:20). The product was obtained as a pale yellow solid in 83.5% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.50 (s, 1H), 8.10 (s, 1H), 7.36 (s, 1H), 6.89 (s, 1H), 3.86 (s, 3H), 3.65 (t, J = 6.5 Hz, 2H), 2.37 (t, J = 7.2 Hz, 2H), 1.72 (dt, J = 14.6, 6.6 Hz, 2H), 1.61 (p, J = 6.7, 6.3 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 175.94, 153.17, 152.77, 152.51, 147.17, 122.59, 116.15, 104.71, 103.29, 56.23, 45.63, 32.08, 25.89, 24.75.

3-(4-Azidobutyl)-7-hydroxy-6-methoxy-4H-chromen-4-one (AMHC-1). A mixture of VI10 (1 equiv, 3 mmol), NaN_3 (1.1 equiv, 3.3 mmol), and anhydrous DMSO (10 mL) was stirred at room temperature. The reaction was monitored by TLC and completed after 8 h. Then, the reaction mixture was poured into cooling water (20 mL) slowly. The solution was extracted with ethyl acetate (30 mL × 3). The organic phase was washed with brine, dried, and concentrated in vacuo, which was purified by silica gel column (PE/EA = 100:1). The product was obtained as a white solid in 91.1% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.50 (s, 1H), 8.09 (s, 1H), 7.35 (s, 1H), 6.88 (s, 1H), 3.86 (s, 3H), 3.34 (s, 2H), 2.35 (s, 2H), 1.54 (s, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.01, 153.25, 152.82, 152.58, 147.24, 122.69, 116.21, 104.78, 103.35, 56.30, 50.95, 28.39, 25.79, 25.08. HRMS (ESI): m/z calcd for $C_{14}H_{16}O_4N_3$ [M + H]$^+$, 290.1135; found, 290.1127.

3-(4-Azidobutyl)-6-methoxy-4-oxo-4H-chromen-7-yl trifluoromethanesulfonate (VI11). To a cooled (0 °C) biphasic mixture of toluene (10 mL), 30% (w/v) aqueous K_3PO_4 (10 mL), and the 7-hydroxychromone analogue AMHC-1 (1 equiv, 2 mmol) was added dropwise triflic anhydride (Tf_2O) (1.5 equiv, 3 mmol) at a rate to maintain the reaction temperature <10 °C. The reaction mixture was allowed to warm to ambient temperature. The reaction was

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monitored by TLC and completed after 5−8 h. The phases were separated, and the aqueous phase was extracted with ethyl acetate (20 mL × 3). The organic phase was washed with brine, dried, and concentrated in vacuo, which was purified by silica gel column (PE/EA = 100:5). The product was obtained as a yellowish-brown solid in 83.4% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.80 (t, J = 0.8 Hz, 1H), 7.76 (s, 1H), 7.39 (s, 1H), 4.01 (s, 3H), 3.36−3.29 (m, 2H), 2.50 (t, J = 4.7 Hz, 2H), 1.67 (p, J = 3.0 Hz, 4H). ^{13}C NMR (101 MHz, chloroform-*d*) δ 176.44, 152.62, 150.10, 149.16, 142.20, 124.00, 123.93, 118.68, 112.99, 107.44, 56.85, 51.22, 28.61, 25.63, 25.45. HRMS (ESI): m/z calcd for $C_{15}H_{15}O_6N_3F_3S$ $[M + H]^+$, 422.06282; found, 422.06314.

7-(Azetidin-1-yl)-3-(4-azidobutyl)-6-methoxy-4H-chromen-4-one (AMAC-1). To a mixture of palladium acetate (0.03 equiv, 3 mol %), BINAP (0.05 equiv, 5 mol %), and cesium carbonate (3 equiv, 3 mmol) were added triflate III11 (1.0 equiv, 1 mmol) and azetidine (1.5 equiv, 1.5 mmol) in toluene. The mixture was stirred for 30 min at room temperature, heated to 80 °C under N_2, and stirred overnight at the same temperature. The reaction mixture was cooled to room temperature, filtered through a Celite pad, and concentrated in vacuo. The residue was dissolved in the mixture solvent THF (10 mL) and 2 N aqueous HCl (10 mL) and stirred at room temperature. The reaction was monitored by TLC and completed after 2−5 h. Then, 2 N aqueous NaOH was added to adjust the pH to 10.0. The solution was extracted with ethyl acetate (30 mL × 3). The organic phase was washed with brine, dried, and concentrated in vacuo, which was purified by silica gel column (PE/EA = 100:30). The product was obtained as a white solid in 53.0% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.59 (s, 1H), 7.36 (s, 1H), 6.13 (s, 1H), 4.10 (t, J = 7.4 Hz, 4H), 3.83 (s, 3H), 3.29 (t, J = 6.5 Hz, 2H), 2.45 (t, J = 6.8 Hz, 2H), 2.33 (p, J = 7.4 Hz, 2H), 1.65 (dt, J = 6.9, 3.1 Hz, 4H). ^{13}C NMR (101 MHz, chloroform-*d*) δ 176.68, 153.55, 150.58, 147.40, 146.51, 122.74, 114.82, 103.74, 98.00, 55.74, 54.06, 51.33, 28.62, 25.84, 25.57, 17.42. HRMS (ESI): m/z calcd for $C_{17}H_{21}O_3N_4$ $[M + H]^+$, 329.1608; found, 329.16208.

General Experimental Procedure for 7-Azido Chromones (AMAC-2, AMAC-3). In a 25 mL round-bottom flask, water (5 mL) and concentrated HCl (2.5 mL) were cooled to 0 °C in an ice bath. 7-Amino chromone (1 equiv, 0.5 mmol) was added, keeping the temperature at 0 °C. A chilled solution of $NaNO_2$ (1.2 equiv, 0.6 mmol) in water (2 mL) was added dropwise. The solution was stirred for 1 h in an ice bath, and then cold saturated $NaHCO_3$ was added to adjust the pH to 7.0. A chilled solution of NaN_3 (1.2 equiv, 0.6 mmol) in water (2 mL) was then added dropwise at the same temperature. The solution was stirred and allowed to come to room temperature and extracted with diethyl ether (20 mL × 3). The combined organic layers were dried over anhydrous Na_2SO_4, filtered, and the solvent was removed under reduced pressure. The crude material was purified by silica gel column (PE/EA = 100:5).

7-Azido-3-propyl-4H-chromen-4-one (AMAC-2). Compound (AMAC-2) was prepared according to the general procedure and the product was obtained as a white solid in 87.3% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 8.20 (dd, J = 8.4, 0.6 Hz, 1H), 7.73 (t, J = 0.8 Hz, 1H), 7.08−7.02 (m, 2H), 2.46−2.39 (m, 2H), 1.60 (h, J = 7.4 Hz, 2H), 0.96 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, chloroform-*d*) δ 177.59, 157.57, 152.44, 145.84, 128.09, 124.86, 120.85, 116.76, 107.51, 27.83, 21.62, 13.90. HRMS (ESI): m/z calcd for $C_{12}H_{12}O_2N_3$ $[M + H]^+$, 230.0924; found, 230.0924.

7-Azido-6-methoxy-3-propyl-4H-chromen-4-one (AMAC-3). Compound (AMAC-3) was prepared according to the general procedure and the product was obtained as a white solid in 84.7% yield. ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.69 (t, J = 0.9 Hz, 1H), 7.57 (s, 1H), 7.01 (s, 1H), 3.96 (s, 3H), 2.42 (td, J = 7.5, 0.9 Hz, 2H), 1.64−1.57 (m, 2H), 0.96 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, chloroform-*d*) δ 176.87, 151.76, 151.50, 149.90, 135.01, 123.94, 121.09, 109.06, 105.72, 56.47, 27.84, 21.57, 13.84. HRMS (ESI): m/z calcd for C13H14O3N3 $[M + H]^+$, 260.1030; found, 260.1030.

General Experimental Procedure for Lys-(7-Amino Chromones) (AMAC-4, AMAC-5). Boc-Lys(Boc)-OH (1 equiv, 0.2 mmol) and 7-amino chromone (1.2 equiv, 0.24 mmol) were dissolved in anhydrous THF (10 mL) at 0 °C, and pyridine (11.0 equiv, 2.2 mmol) was added. After stirring for 30 min, $POCl_3$ (3.7 equiv, 0.74 mmol) was added dropwise and the reaction mixture was stirred at 0 °C for 100 min and then for 60 min at room temperature. The reaction mixture was poured into a mixture of ice and saturated aq $NaHCO_3$ (1:1, v/v, 20 mL), then concentrated to approximately 20 mL and extracted with EtOAc (3 × 20 mL). The organic phase was then washed with aq HCl (1 N, 2 × 20 mL) and saturated aq $NaHCO_3$ (20 mL) and brine (20 mL), dried over $MgSO_4$ and purified by silica gel column (PE/EA = 100:30) affording Boc-Lys(Boc)-(7-amino chromone) as a white solid. Boc-Lys(Boc)-(7-amino chromone) was dissolved in 4.0 M hydrogen chloride solution in dioxane (5 mL), and the reaction mixture was stirred at r.t. for 30 min. The reaction mixture evaporated to dryness, affording Lys-(7-amino chromone) hydrochloride.

2,6-Diamino-N-(4-oxo-3-propyl-4H-chromen-7-yl)hexanamide (AMAC-4). Compound (AMAC-4) was prepared according to the general procedure and the product was obtained as a white solid in 65.1% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.77 (s, 1H), 8.52 (s, 2H), 8.20 (s, 1H), 8.09 (s, 1H), 8.03 (d, J = 8.7 Hz, 1H), 7.98 (s, 2H), 7.68 (d, J = 8.6 Hz, 1H), 4.18 (s, 1H), 2.88−2.69 (m, 2H), 2.33 (t, J = 7.1 Hz, 2H), 1.89 (ddt, J = 20.5, 13.3, 7.0 Hz, 2H), 1.61 (dt, J = 13.7, 7.0 Hz, 2H), 1.50 (dq, J = 20.6, 7.1 Hz, 4H), 0.90 (t, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.30, 168.99, 156.97, 153.67, 143.36, 126.50, 123.86, 119.66, 117.31, 107.22, 53.24, 38.61, 30.73, 27.48, 26.70, 21.64, 21.58, 14.12. HRMS (ESI): m/z calcd for $C_{18}H_{26}O_3N_3$ $[M + H]^+$, 332.1969; found, 332.1972.

2,6-Diamino-N-(6-methoxy-4-oxo-3-propyl-4H-chromen-7-yl)-hexanamide (AMAC-5). Compound (AMAC-5) was prepared according to the general procedure and the product was obtained as a white solid in 62.7% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.29 (s, 1H), 8.47 (s, 2H), 8.36 (s, 1H), 8.20 (s, 1H), 7.92 (s, 2H), 7.51 (s, 1H), 4.36 (s, 1H), 3.99 (s, 3H), 2.82−2.71 (m, 2H), 2.39−2.30 (m, 2H), 1.90−1.77 (m, 2H), 1.62−1.56 (m, 2H), 1.52 (dt, J = 15.1, 7.6 Hz, 2H), 1.47−1.37 (m, 2H), 0.89 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.01, 169.49, 153.60, 151.15, 147.46, 132.60, 123.21, 119.71, 109.79, 104.34, 56.86, 52.97, 38.73, 31.07, 27.56, 26.93, 21.68, 21.60, 14.09. HRMS (ESI): m/z calcd for $C_{19}H_{28}O_4N_3$ $[M + H]^+$, 362.2074; found, 362.2074.

Cell Viability Assay. Human colon cancer cell HCT-116, human liver cancer cell HepG2, and human gastric cancer cell BGC-823 were obtained from the cell center of Chinese Academy of Medical Sciences & Peking Union Medical College. They were cultured in Dulbecco's modified Eagle's medium (DMEM) medium (Invitrogen) with 10% fetal bovine serum (Gibco) at 37 °C with 5% CO_2. Four compounds (III2, III5, VI2, and VI5) were selected and assessed that the cytotoxicity, IC_{50}, was calculated following the standard protocol.[4]

Measurement of Various Photophysical Properties.[42] Target compounds that have been synthesized were further measured for fluorescence quantum yield, molar extinction coefficient, excitation and emission spectra, and fluorescence lifetime. UV−visible spectra were acquired with a Shimadzu UV-2700, UV−vis spectrophotometer. Fluorescence quantum yield and excitation and emission spectra were measured and calculated with a HITACHI F-7000 fluorescence spectrophotometer. Fluorescence lifetime was measured with Edinburgh Analytical Instruments F900. To obtain fluorescence excitation and emission spectra, we dissolved the target compound in 0.1 M Tris−HCl buffer containing 0.1% DMSO, pH 8.0, at a concentration of 10 μM and measured using a HITACHI F-7000 fluorescence spectrophotometer. A suitable emission wavelength was set to obtain the excitation spectra. Meanwhile, a suitable excitation wavelength was set to obtain the emission spectra.

Fluorescence quantum yield was recorded using a comparative method of Williams et al. The detection was carried out in 0.1 M Tris−HCl buffer (pH 8.0) at a suitable concentration at which the absorption below 0.05 occurs at the maximum excitation wavelength using quinine sulfate (0.5 μg/L in 0.1 M H_2SO_4, Φ = 0.54) as a reference. The quantum yield was calculated using the following equation

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$$\Phi_x = \Phi_{st}\left(\frac{A_{st}F_x}{A_xF_{st}}\right)\left(\frac{n_x}{n_{st}}\right)^2$$

where the subscripts x and st denote test and standard, respectively, Φ is the fluorescence quantum yield, A is the absorbance at the maximum excitation wavelength, F is the area under the emission curve, and n is the refractive index of the solvents used. For the tested compounds and the standard, the excitation wavelength was at 345 nm while keeping the absorption below 0.05.

Absorbance maximum wavelength, molar extinction coefficient at absorbance max, and excitation, emission maximum wavelength of each compound (10 μM) were also measured in aqueous buffer, 0.1 M Tris−HCl, pH 8.0.

Values of compound lifetimes were obtained using the Edinburgh Analytical Instruments F900.

EdU Labeling with AMHC-1 and AMAC-1. All animal experiments were conducted in compliance with the Care and Use of Laboratory Animals with the approval of Peking Union Medical College and Chinese Academy of Medical Sciences' Animal Studies Committee. AMHC-1 and AMAC-1 were used in this section with a final concentration of 10 μM in PBS buffer (pH 7.0 and 9.0). For more details on this part, please refer to ref 17.

Application of AMAC-2 and AMAC-3 to Detect Hydrogen Sulfide. *Reaction Selectivity of AMAC-2 and AMAC-3 toward Hydrogen Sulfide.* Fluorescence responses for the AMAC-2 or AMAC-3 probe was measured (For AMAC-2, E_x = 317 nm and E_m = 442 nm. For AMAC-3, E_x = 333 nm and E_m = 432 nm) at 5, 15, 35, 65, and 105 min. All assays were performed in 0.1 M Tris−HCl buffer at pH 8.0 and measured with the EnSpire Multimode plate reader from PerkinElmer.

GSH: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 1 mM glutathione in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

Cys: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 1 mM cysteine in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

SO_3^{2-}: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 1 mM Na_2SO_3 in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

HSO_3^-: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 1 mM $NaHSO_3$ in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

$S_2O_3^{2-}$: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 1 mM $Na_2S_2O_3$ in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

$S_2O_5^{2-}$: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 1 mM $Na_2S_2O_5$ in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

Lipoic acid: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 1 mM lipoic acid in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

HSO_4^-: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 1 mM $NaHSO_4$ in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

SO_4^{2-}: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 1 mM Na_2SO_4 in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

NO_2^-: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 1 mM $NaNO_2$ in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

L-Ascorbic acid salt: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 1 mM L-ascorbic acid salt in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

HS^-: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 500 μM NaHS in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

Fe^{2+}: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 1 mM $FeSO_4$ in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

NO: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 1 mM $Na_2Fe(CN)_5NO{\cdot}2H_2O$ in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

H_2O_2: 1 μL of a 1 M stock solution of H_2O_2 in 0.1 M Tris−HCl buffer at pH 8.0 was added to 1 mL of 10 μM AMAC-2 or AMAC-3 in 0.1 M Tris−HCl buffer at pH 8.0 with 0.1% DMSO and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

TBHP: 1 μL of a 1 M stock solution of *tert*-butyl hydroperoxide in 0.1 M Tris−HCl buffer at pH 8.0 was added to 1 mL of 10 μM AMAC-2 or AMAC-3 in 0.1 M Tris−HCl buffer at pH 8.0 with 0.1% DMSO and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

OCl^-: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO was added to 1 mL of 1 mM NaClO in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

ClO_4^-: 1 μL of a 1 M stock solution of $Fe(ClO_4)_2$ in 0.1 M Tris−HCl buffer at pH 8.0 was added to 1 mL of 10 μM AMAC-2 or AMAC-3 in 0.1 M Tris−HCl buffer at pH 8.0 with 0.1% DMSO and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

$^1O_2$: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO and 1 μL of a 2 M stock solution of H_2O_2 in 0.1 M Tris−HCl buffer at pH 8.0 were added to 1 mL of 1 mM NaClO in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

$^{\bullet}OH$: 1 μL of 10 mM AMAC-2 or AMAC-3 in DMSO and 1 μL of a 2 M stock solution of H_2O_2 in 0.1 M Tris−HCl buffer at pH 8.0 were added to 1 mL of 1 mM $FeSO_4$ in 0.1 M Tris−HCl buffer at pH 8.0 and the mixture was vortexed for 10 s and then transferred to a 96-well plate.

Time-Dependent Emission Spectra of AMAC-2 or AMAC-3 in the Presence of NaHS. A 10 μM solution of AMAC-2 or AMAC-3 in 0.1 M Tris−HCl buffer at pH 8.0 was prepared from a 10 mM stock solution of AMAC-2 or AMAC-3 in DMSO in a 1.5 mL Eppendorf tube. Then, 10 μL of 10 mM stock solution of NaSH in 0.1 M Tris−HCl buffer at pH 8.0 was added (for a final concentration of 100 μM) and the mixture was vortexed for 10 s and then transferred to a cuvette. For AMAC-2, emission spectra (E_x = 317 nm, E_m = 340−700 nm) were collected at 5, 15, 35, 65, 105, and 145 min. For AMAC-3, emission spectra (E_x = 333 nm, E_m = 360−700 nm) were collected at 5, 15, 30, 50, 75, 105, and 145 min. The spectrum at t = 0 was acquired from a 10 μM solution of AMAC-2 or AMAC-3 without the addition of NaSH. All spectra were measured with a HITACHI F-7000 fluorescence spectrophotometer.

Linear Correlation between the Fluorescence Intensity and NaHS Concentrations. A 10 μM solution of AMAC-2 or AMAC-3 in 0.1 M Tris−HCl buffer at pH 8.0 was prepared from a 10 mM stock solution of AMAC-2 or AMAC-3 in DMSO in a 25 mL Eppendorf tube. Then, 1 μL of 0, 1, 1.25, 2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mM stock solution of NaSH in 0.1 M Tris−HCl buffer at pH 8.0 was incubated with 1 mL of 10 μM solution of AMAC-2 or AMAC-3 in 0.1 M Tris−HCl buffer at pH 8.0 at room temperature for 30 min, respectively, in 1.5 mL Eppendorf tubes, and then transferred to a 96-well plate and measured with the EnSpire Multimode plate reader from PerkinElmer.

Enzyme Kinetic Study of Trypsin toward AMAC-4 and AMAC-5. A total of 2, 10, 20, 100, 200, 400, 800, and 1600 μM of probe AMAC-4 or AMAC-5 in 0.1 M Tris−HCl buffer at pH 8.0 and 100 U/mL trypsin in 0.1 M Tris−HCl buffer at pH 8.0 were mixed in equal volumes, respectively, in a 96-well plate and instantly measured

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every 2 min for 60 min with the EnSpire Multimode plate reader from PerkinElmer (For AMAC-4, E_x = 317 nm and E_m = 442 nm. For AMAC-5, E_x = 333 nm and E_m = 432 nm).

Docking Study of AMAC-4 and AMAC-5 in the Active Center of Trypsin.[54] The docking study was performed to investigate the binding mode of compounds A and B by AutoDock 4.2.6 (Scripps Research Institute, San Diego, CA). The X-ray crystallographic structure of the trypsin complex with guanidine-3-propanol (PDB Code: 2A31) was downloaded from the Protein Data Bank (PDB) and prepared by the removal of water and addition of hydrogen.[53] The original ligand guanidine-3-propanol was removed from the PDB file. The files of protein and molecules were converted to pdbqt format by AutoDock. A grid box with dimensions of 40 × 40 × 40 Å^3 (33.540, 27.993, 13.366) with a spacing of 0.375 Å was constructed around the docking area using Autogrid 4.2 software. The docking procedure was carried out for the unchanged conformation of the receptor and flexible ligand molecules. The lowest energy conformations were selected, and the ligand interactions with trypsin were determined.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c01508.

^{1}H and ^{13}C NMR spectra for all final compounds (I1−7, II1−7, III1−9, IV1−8, V1−8, VI1−9, AMHC-1, AMAC-1, AMAC-2, AMAC-3, AMAC-4, and AMAC-5); the HPLC traces of compounds (VI1, VI2, and VI5) (PDF)

Molecular formula strings (CSV)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BODIPY, boron-dipyrromethene; BINAP, 2,2′-bis-(diphenylphosphino)-1,1′-binaphthyl; EA, ethyl acetate; EdU, 5-ethynyl-2′-deoxyuridine; PE, polyethylene; LOD, limit of detection; TCT, 2,4,6-trichloro-1,3,5-triazin; THF, tetrahydrofuran; TICT, twisted internal charge transfer; TRP, tryptophan

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