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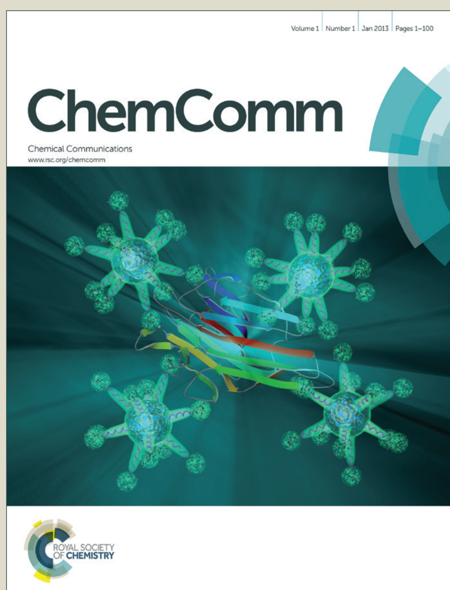

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Development of 3-Alkyl-6-Methoxy-7-Hydroxy-Chromone (AMHC) from Natural Isoflavones, a New Fluorescent Scaffold for Biological Imaging

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Starting from 7-hydroxyisoflavones, we developed a new class of fluorescent scaffold, 3-Alkyl-6-Methoxy-7-Hydroxy-Chromone (AMHC, MW~205.19, λ_{ab}~350 nm, λ_{em}~450 nm) via a trial and error process. AMHCs have the advantages of being a small molecular moiety, having strong fluorescence in basic buffers, reasonable solubility and stability, non-toxicity, and are conveniently linked to pharmacophores. AMHCs were successfully used in fluorescence microscopy imaging of cells and tissues.

Fluorescent dyes have been widely used in biological research for analytical sensing and biological imaging.[1-9] In spite of increasing demand, the library of conventional fluorophores only contains a limited number of scaffolds, including naphtalimide, styryl, xanthone, coumarin, dapoxyl, BODIPY, rhodol and tricarbocyanine.[3,4,7,8] Efforts to diversify the fluorophore library, include both the de novo synthesis of new scaffolds and the optimization of known scaffolds.[8,10-17] However, due to the complex of photophysical properties of fluorophores, it is difficult to predict the emission wavelength or quantum yield of a fluorogenic scaffold, and most of these works are performed empirically.[8,10-19]

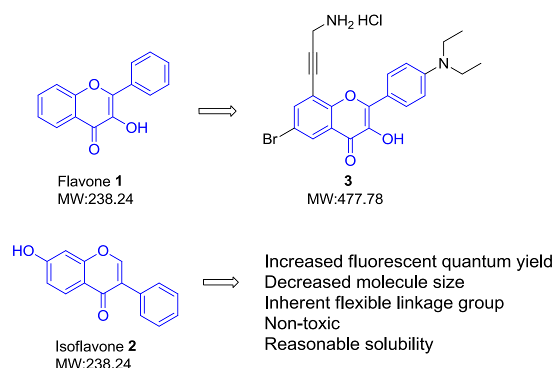

Fig 1.Previous development of flavone **1** based fluorophore **3** and the desired optimization of isoflavone **2** in this study.

Natural products are important source of new scaffolds for fluorophores.[1,20] Natural 7-hydroxyisoflavones and synthetic 3-hydroxyflavones have been reported to have weak fluorescence in biological buffers. This limits their application in biological imaging (Figure S1, S2).[21-29] Efforts have been made to circumvent these problems. For example, 3-hydroxyflavone **1** has been further developed to a series of moderate to strong fluorophores in ethanol, such as **3** (Φ= 0.48 in 95% ethanol) (Figure 1). This optimisation has increased the fluorescence, but the size of the molecule was also increased.[30,31]

To our knowledge, 7-hydroxyisoflavone **2** has not yet been used as a lead for the development of new fluorophores. Nevertheless, 7-hydroxyisoflavone is able to exhibit fluorescence, albeit weakly in a biological buffer, and has superior aqueous solubility compared to 3-hydroxyflavone (Figure S3a, S3b). Thus, in this study we opted to optimize the photophysical properties of isoflavones in order to develop of a new class of fluorescent dyes.

Considering the difficulty of theoretical prediction of optical properties,[1] our optimisation was carried out by trial and error. We investigated the effect of various substituents at different positions of the isoflavone core upon its photophysical properties. Subsequently, favourable modifications were combined to design new fluorophores. In addition, given the future application for biological imaging, possible properties were also designed into final fluorophores.

During our first round of optimisation, we developed a specific library of isoflavone analogues via chemistry synthesis.[32] After fully evaluating the fluorescent properties of the isoflavones, a structure fluorescence relationship was observed (Figure 2, S4-S8):

- 7-position: The isoflavones with 7-OH are often fluorogenic. Acetylation, or methylation of the 7-OH quenched the fluorescence of the isoflavone core (Figure S4).
- 6-position: 6-OMe alone is able to improve the fluorescence quantum yield (Φ=0.21) of the isoflavone core. Other substituents at this position (OH, CH_3) do not have an effect (Figure S5a, S5b). In addition, 6-OMe substitution marginally affects the fluorescence quantum yield of 7-hydroxyisoflavone.
- 5-position: 7-Hydroxyisoflavones containing 5-hydroxyl are non-fluorogenic. Previous studies elucidated that 5-hydroxylflavone

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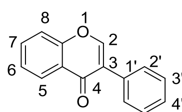
undergo the excited state intra-molecular proton transfer (ESIPT) and result in the low fluorescence quantum yield (Figure S6).[25-27]

● 8-position: Substitution at the 8-positionis is not favourable to increase the fluorescence quantum yield of 7-hydroxyisoflavone. In addition, 8-OH abolishes fluorescence of 7-hydroxyisoflavone (Figure S7).

● 3-position: For the 7-hydroxyisoflavone core, the *2'-, 3'-,* and *4'*-positions of the 3-phenyl ring were substituted with various groups to evaluate the effect on fluorescence. The results show that electro-donating *2'*-substituents can increase the fluorescence quantum yield of 7-hydroxyisoflavone (CH_3>OMe>OH) (Figure S8).



* 7-OH is critical to obtain fluorescence
* 6-OMe alone can activate fluorescence
* 5-OH quenches fluorescence generated by 7-OH
* 8-Substitution is not preferred by 7-OH related fluorescence
* *2'*-Substitution increases 7-OH related fluorescence

Fig 2. The key trend of structure fluorescence relationship of isoflavone derivatives in 0.1M Tris-HCl, pH8.0.

Next, we investigated the importance of the 3-phenyl group toward the fluorescence of 7-hydroxyisoflavone. 3-Alkyl-7-hydroxy-chromones were synthesized to evaluate their fluorescence. Interestingly, 3-methyl-7-hydroxychromone showed an increased fluorescence quantum yield compared to 3-phenyl-7-hydroxychromone (Figure S9a, S9b). For comparison, we also synthesized and evaluated 2-methyl-7-hydroxychromone, and found that this compound exhibits an identical fluorescence quantum yield (Φ=0.21) to 3-methyl-7-hydroxychromone (Φ=0.21) (Table S2, Figure S10). Thus, further optimisation was focused on 3-alkyl chromone derivatives.

3-Alkyl-7-hydroxychromone has increased fluorescence quantum yield and improved aqueous solubility. Interestingly, the 3-alkyl chain serves as a natural spacer, and can be designed with a terminal active group for attachment to a pharmacophore without seriously affecting the photophysical properties of the fluorophore. However, so far the fluorescence quantum yield of 3-alkyl-7-hydroxychromone is only ~0.20 in 0.1M Tris-HCl buffer, pH 8.0 (Table 1,Table S2), which is relatively poor for use as a fluorescent sensor in biological studies.

First round of optimisation (Figure 2) demonstrated that 6-OMe alone activates the fluorescence of the 3-phenylchromone core. Thus, a 6-OMe substitution was also incorporated into the 3-alkyl-7-hydroxychromone core to afford a new scaffold, 3-alkyl-6-methoxy-7-hydroxy-chromone (AMHC). Intriguingly, AMHCs exhibited a dramatically increased fluorescence quantum yield of around 0.5 in 0.1M Tris-HCl, pH 8.0, and reasonable extinction coefficients (ε) in the range of (1.0-2.0) $\times 10^5$ M^{-1} cm^{-1}. These result in fluorophore brightnesses of ~ 10^5 M^{-1} cm^{-1}, which is sufficient to be used as fluorescent sensors (Table 1). In addition, five of the representative fluorophores had their fluorescence lifetimes measured (Table 1). For lifetime studies the excitation wavelength was set at λ_{ex} = 375nm, with all of the tested compounds showing a lifetime in the range of 1.7-5.2 ns. Our development has improved the fluorescence lifetime of AMHCs compared to that of natural isoflavone **6**, which makes AMHCs suitable as fluorescent probes in biological buffers.

Table 1. The photophysical properties of selected compounds.

Compound[a]	ε_{max}[b]	λ_{ab}[c]	λ_{em}[c]	Φ[d]	τ[e]
6	12600	311	468	0.05	1.72
34	7300	288	419	0.21	5.24
70	12700	334	454	0.21	2.11
81	16400	345	447	0.48	4.49
83	16300	346	445	0.41	4.31

[a]Measurements were made in 0.1M Tris-HCl duffer, pH8.0. [b]Unit M^{-1} cm^{-1}. [c]Unit nm. [d]Determined with quinine sulfate (Φ =0.54, 0.1M H_2SO_4) as reference.[33] [e]Unit ns. For details, please see supporting information.

The development process of AMHC fluorophores is presented in Figure 3. The fluorescence brightness was gradually increased during optimisation. The commercial fluorescent dye 7-amino-4-methylcoumarin (AMC) was chosen for comparison since AMC has a similar wavelength range of excitation and emission maximum as the AMHCs. Interestingly, under a monochrome camera (Figure 3A), AMHC fluorescent dyes show more intensive brightness than that of AMC tested under identical conditions. Given that monochrome photography is commonly used in fluorescence microscopy,[34,35] this beneficial property enhances the potential of AMHC sensors to be used in biological imaging. We also imaged the fluorescence with a colour digital camera (Figure 3B), and AMHC dyes show a similar colour intensity as AMC. The discrepancy between the brightness of AMHC and AMC under monochrome and colour cameras is likely caused by the different sensor systems of monochrome and colour cameras. Also, the optical behaviors of AMHCs and AMC are dependent upon their different solvent micro-environments.[36,37,38]

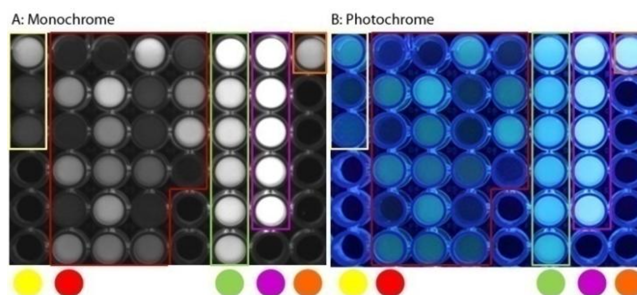


Fig3. Screening fluorescence intensity of selected compounds (100 μM) in 0.1M Tris-HCl, pH 8.0 in a microtiter plate. Yellow label, natural products of isoflavones; Red label, 3-phenyl-7-hydroxychromone

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analogues; Green label, 3-alkyl-7-hydroxychromone derivatives; Purple label, 3-alkyl-6-methoxy-7-hydroxychromone derivatives; Orange label: AMC. For details of compounds, please see table S1.

Natural 7-hydroxyisoflavones were reported to show a pH dependent fluorescence effect.[21-29] Thus, the fluorescence of AMHCs were also evaluated at different pH values (Figure 4). Compound **6** (natural isoflavone) and **81** (AMHC derivative) were dissolved in methanol:water (1:1) with the pH ranging from 1 to 12. Both **6** and **81** demonstrated pH dependent fluorescence effects, but fluorophore **6** only showed moderately high fluorescence intensity at the pH range of 9-11, with the fluorescence dropping at pH 12. While, fluorophore **81** exhibited a moderate fluorescence intensity from pH 5-8, which will enable it to be used in biological imaging. Moreover, **81** possesses an exceptional fluorescence intensity above pH 9. This demonstrates that AMHCs are a class of fluorophores with strong fluorescence properties.

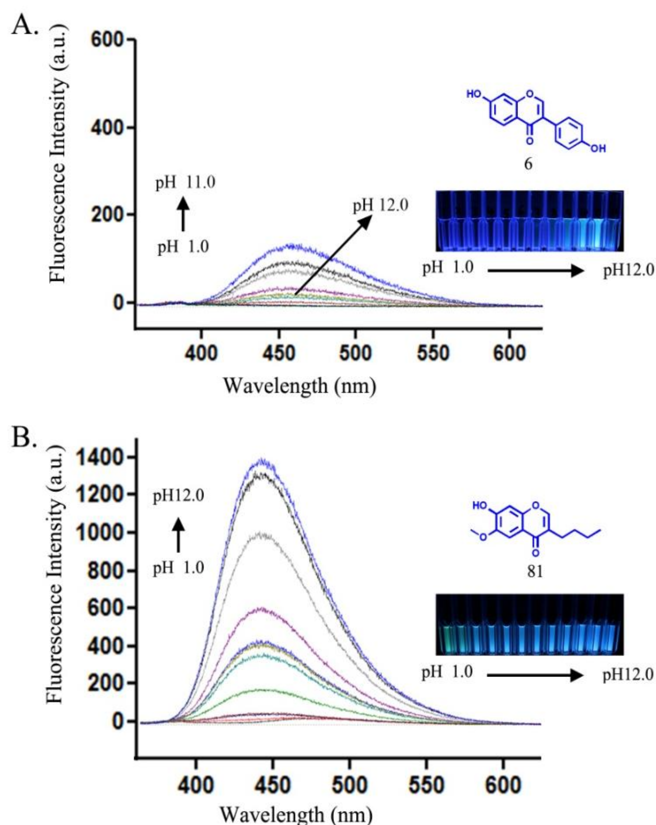

Fig4. Fluorescence emissions of **6** and **81** show variation over different pHs at the concentration of 10 μM (E_x=350nm). A. Fluorescence emission spectra and fluorescence intensity of **6** at pH ranging from 1 to 12; B. Fluorescence emission spectra and fluorescence intensity of **81** at pH ranging from 1 to 12.

AMHCs were further evaluated for their suitability as biological reagents. Several AMHC analogues were screened for cell toxicity and found not show any toxicity at 50 μM (Table S4). In addition, AMHCs are soluble in 0.1M Tris-HCl buffer, pH 8.0 at a concentration of 100 μM.

EdU (5-ethynyl-2'-deoxyuridine) incorporates into DNA and can be used to detect cell proliferation.[39-41]A fluorescent dye that contains an azide group can label EdU via click chemistry. Therefore, we synthesized AMHC dye **83** (Table 1), which contains an azide group at the end of the alkyl chain. HepG2 cells were incubated with 10 μM EdU, followed by the addition of 5 μM **83** in a click chemistry reaction buffer, in order to label EdU incorporated to DNA. During this experiment we observed a strong blue fluorescence at the HepG2 cell nucleus (Figure 5: A, B, C). It has previously been reported that the commercial fluorescein-derived dyes Alexa488-azide and Alexa594-azide (Molecular Probes®) stain rat bone marrow cells in the absence of EdU (false-positive staining).[41] Therefore, the same rat bone marrow cell staining experiment was performed with both Alexa488-azide and **83** in the absence of EdU. We were able to observe the same false staining of rat bone marrow cells by Alexa488-azide, but not by AMHC dye **83** (Figure S13). This shows an additional advantage of the AMHC scaffold dyes. In addition, this false-positive staining case also enhances the necessity to develop fluorophores with varied scaffolds.

AMHCs were also evaluated for their application in tissue staining. Fluorescence imaging of tissue samples requires fluorescent dyes with a strong fluorescence intensity due to the high fluorescence background often observed in tissues. We implanted lewis lung cancer cells in adult female C57BL mice, and 10 days later we treated the mice with EdU. Subsequently, 5 μM of **83** in a click chemistry reaction buffer was used to label EdU in mice tumor tissue. The tumor tissue was imaged under a fluorescent microscope, and a strong and specific blue fluorescence at cell nucleus was observed (Figure 5: D, E, F).

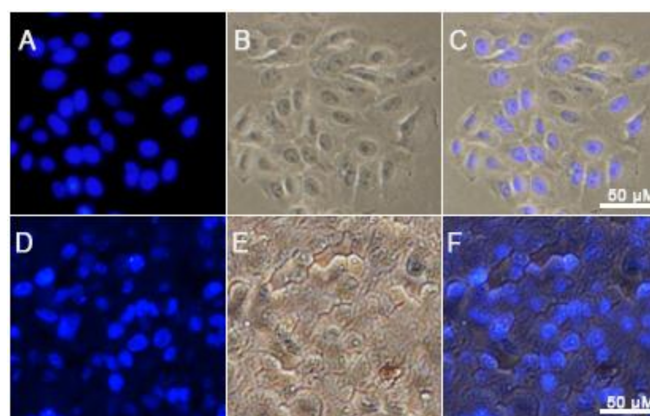

Fig5. Application of AMHC dye to label and image EdU that was incorporated into DNA in cultured cells (A-C) and mice tumor tissues (D-F). A: **83** detected EdU in cell nucleus of HepG2 cells, B: HepG2 cell imaged in bright field, C: Merged image of A and B. D: **83** detected EdU in cell nucleus of tumor tissue, E: tumor tissue imaged in bright field, F: Merged image of D and E.

Conclusions

Starting with the natural 7-hydroxyisoflavone, we prepared a number of isoflavone derivatives with optimized fluorescence properties. During this process we discovered a new class of fluorogenic scaffold, 3-alkyl-6-methoxy-7-hydroxychromones (AMHC). The fluorophores of AMHCs were found to possess good fluorescent photophysical properties, as well as reasonable stability and solubility in biological buffer. Moreover, the AMHC scaffold contains an alkyl chain, which enables the design of various terminal linker systems. AMHCs were successfully used in cellular and tissue imaging experiments. Given the novelty of this fluorescent scaffold, and its advantageous photophysical properties, we envisage that

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AMHCs can be used to design various biological probes, or used as a building block to develop new fluorophores with other desired properties.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [Experimental procedures, photophysical properties, compounds data]. See DOI: 10.1039/c000000x/

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