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Development of 3-alkyl-6-methoxy-7-hydroxychromones (AMHCs) from natural isoflavones, a new class of fluorescent scaffolds for biological imaging⁺

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Starting from 7-hydroxyisoflavones, we developed a new class of fluorescent scaffolds, 3-alkyl-6-methoxy-7-hydroxy-chromones (AMHCs, $M_{\rm W} \sim 205.19$, $\lambda_{\rm ab} \sim 350$ nm, $\lambda_{\rm em} \sim 450$ 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 the increasing demand, the library of conventional fluorophores only contains a limited number of scaffolds, including naphthalimide, 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 studies are performed empirically.^{8,10–19}

Natural products are important sources 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 (Fig. S1 and S2, ESI[†]).^{21–29} Efforts have been made to circumvent these problems. For example, 3-hydroxyflavone **1** has



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

been further developed into a series of moderate to strong fluorophores in ethanol, such as 3 (Φ = 0.48 in 95% ethanol) (Fig. 1). This optimisation has increased the fluorescence, and 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 (Fig. S3a and b, ESI†). Thus, in this study we opted to optimize the photophysical properties of isoflavones in order to develop a new class of fluorescent dyes.

Considering the difficulty in theoretical prediction of optical properties,¹ our optimisation was carried out by a trial and error process. We investigated the effect of various substituents at different positions of the isoflavone core on 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* chemical synthesis.³² After fully evaluating the fluorescence properties of the

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- * 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 0.1 M Tris-HCl, pH 8.0.

isoflavones, a structure fluorescence relationship was observed (Fig. 2 and Fig. S4–S8, ESI[†]):

• 7-Position: the isoflavones with 7-OH are often fluorogenic. Acetylation or methylation of 7-OH quenched the fluorescence of the isoflavone core (Fig. S4, ESI[†]).

• 6-Position: 6-OMe alone is able to improve the fluorescence quantum yield ($\Phi = 0.21$) of the isoflavone core. Other substituents at this position (OH and CH₃) do not have an effect (Fig. S5a and b, ESI†). In addition, 6-OMe substitution marginally affects the fluorescence quantum yield of 7-hydroxyisoflavone.

• 5-Position: 7-hydroxyisoflavones containing 5-hydroxyl are nonfluorogenic. Previous studies elucidated that 5-hydroxylflavone undergoes the excited state intra-molecular proton transfer (ESIPT) and results in the low fluorescence quantum yield (Fig. S6, ESI[†]).²⁵⁻²⁷

• 8-Position: substitution at the 8-position is not favourable to increase the fluorescence quantum yield of 7-hydroxyisoflavone. In addition, 8-OH abolishes the fluorescence of 7-hydroxyisoflavone (Fig. S7, ESI†).

• 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 electron-donating 2'-substituents can increase the fluorescence quantum yield of 7-hydroxyisoflavone (CH₃ > OMe > OH) (Fig. S8, ESI⁺).

Next, we investigated the importance of the 3-phenyl group in 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 (Fig. S9a and b, ESI†). For comparison, we also synthesized and evaluated 2-methyl-7-hydroxychromone, and found that this compound exhibits an identical fluorescence quantum yield ($\Phi = 0.21$) to 3-methyl-7-hydroxychromone ($\Phi = 0.21$) (Table S2 and Fig. S10, ESI†). 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 0.1 M Tris-HCl buffer, pH 8.0 (Table 1 and Table S2, ESI†), which is relatively poor for use as a fluorescent sensor in biological studies.

Table 1 The photophysical properties of selected compounds

1 1 3						
Compound ^{<i>a</i>}	$\varepsilon_{\max}{}^{b}$	λ_{ab}^{c}	$\lambda_{\rm em}{}^c$	Φ^d	τ^e	
	12 600	311	468	0.05	1.72	
	7300	288	419	0.21	5.24	
HO 70 ⁰	12 700	334	454	0.21	2.11	
HO O 81 ⁰	16 400	345	447	0.48	4.49	
HO O N ₃	16300	346	445	0.41	4.31	
83						

^{*a*} Measurements were made in 0.1 M Tris-HCl buffer, pH 8.0. ^{*b*} Unit M^{-1} cm⁻¹. ^{*c*} Unit nm. ^{*d*} Determined with quinine sulfate ($\Phi = 0.54$, 0.1 M H₂SO₄) as given in ref. 33. ^{*e*} Unit ns. For details, please see ESI.

The first round of optimisation (Fig. 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 0.1 M Tris-HCl, pH 8.0, and reasonable extinction coefficients (ε) in the range of (1.0-2.0) \times 10⁵ M⁻¹ cm⁻¹. This results in a fluorophore brightness of $\sim 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, 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} = 375 nm, 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.

The development process of AMHC fluorophores is presented in Fig. 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 to AMHCs. Interestingly, under a monochrome camera (Fig. 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 (Fig. 3B), and AMHC dyes show similar colour intensity to 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



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

of AMHCs and AMC are dependent on their different solvent micro-environments. $^{36\mathchar`-38}$

Natural 7-hydroxyisoflavones were reported to show a pH dependent fluorescence effect.^{21–29} Thus, the fluorescence of AMHCs was also evaluated at different pH values (Fig. 4). Compounds **6** (natural isoflavone) and **81** (AMHC derivative) were dissolved in methanol:water (1:1) with the pH ranging



Fig. 4 Fluorescence emission of **6** and **81** show variation over different pH values at the concentration of 10 μ M (E_x = 350 nm). (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.

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 moderate fluorescence intensity from pH 5 to 8, which will enable it to be used in biological imaging. Moreover, **81** possesses exceptional fluorescence intensity above pH 9. This demonstrates that AMHCs are a class of fluorophores with strong fluorescence properties.

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

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 into DNA. During this experiment we observed a strong blue fluorescence at the HepG2 cell nucleus (Fig. 5A-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 using 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 (Fig. S13, ESI⁺). 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



Fig. 5 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 the cell nucleus of tumor tissue, (E) tumor tissue imaged in bright field, (F) merged image of D and E.

fluorescent dyes with 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 μ M 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 (Fig. 5D–F).

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 scaffolds, 3-alkyl-6-methoxy-7-hydroxychromones (AMHCs). The fluorophores of AMHCs were found to possess good fluorescence 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 AMHCs can be used to design various biological probes, or used as building blocks to develop new fluorophores with other desired properties.

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