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Synthesis of 3-hydroxyflavone fluorescent probes and study of their fluorescence properties

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Abstract

Direct measurement of dipole potential in biological membranes has been impossible and 3-hydroxyflavones (3HFs) have allowed detection of changes in dipole potential in biological systems. In the present study, sixteen derivatives of 3HF with aliphatic hydrocarbon chains of different lengths at 4′-position and 6-position were synthesized. The basic fluorescence properties of 3HFs are maintained in all the probes in terms of strong blue shift in maximum fluorescence emission wavelength and >100 fold increase in quantum yield in organic solvents and in dioleoylphosphatidylcholine (DOPC) small unilamellar vesicles (SUV) in comparison to in aqueous Hepes buffer (15 mmol/L, pH 7.4). More importantly, the ability of the new compounds to report dipole potential changes in biological systems are also maintained, since all the new probes showed spectrum properties that are similar to yet different from that of **F4N1**, which potentially may allow more sensitive measurement of the dipole potential change in membranes.

Keywords: 3-Hydroflavones (3HFs); Fluorescent probes; ESIPT; Dipole potential

3-Hydroxyflavones (3HFs) are dual-band fluorescent dyes due to an excited state intramolecular proton transfer (ESIPT) reaction. The dual emission of 3HF is highly sensitive to the properties of the environment, such as polarity and H-bond donor and acceptor ability. Therefore, they have been used as highly sensitive probes of their microenvironments in biological systems [1,2]. It has been found that the introduction of an electron donor group to the 4′-position of the 3HF increased the sensitivity of fluorescence, and that the attachment of positively charged ammonium group at 6-position of the 3HF dramatically changed the relative intensities of the two emission bands [3]. Accordingly, in the present study, two series, sixteen 3HFs derivatives including 14 new ones, were synthesized, and the effect of aliphatic chains of different lengths at 4′- and 6-positions of 3HFs on the fluorescence properties were studied in organic solvents and in dioleoylphosphatidylcholine (DOPC) small unilamellar vesicles (SUV).

1. Experimental

F2 and F4 two series, sixteen 3HFs derivatives including fourteen new ones, were synthesized according to the synthetic route shown in Scheme 1.

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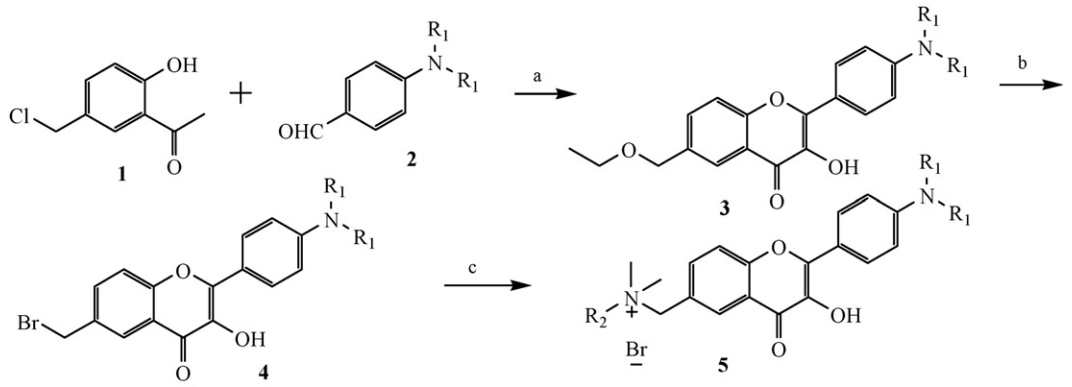

Scheme 1. The synthetic route of compounds **5**: $R_1 = n\text{-}C_2H_5$, or $n\text{-}C_4H_9$; $R_2 = CH_3$, $n\text{-}C_4H_9$, $n\text{-}C_6H_{13}$, $n\text{-}C_7H_{15}$, $n\text{-}C_8H_{17}$, $n\text{-}C_9H_{19}$, $n\text{-}C_{10}H_{21}$, $n\text{-}C_{12}H_{25}$. Condition and reagent: (a) H_2O_2, NaOH, EtOH, 0–60 °C; (b) 40% HBr, reflux 3 h; (c) *N,N*-dimethylalkylamine, EtOH, reflux 8 h.

Compound **1** and **2** were prepared according to the published methods [1,4]. Compound **3** with $R_1 = n\text{-}C_2H_5$ or $n\text{-}C_4H_9$ at 4′-position, having the 3-hydroxyflavone core, were synthesized as the key intermediates using a modification of Algar-Flynn-Ayamada reaction [5]. After the intermediates were bromided by 40% HBr to give compound **4**, the fluorescent probes (compound **5**) could be obtained following the reaction with *N,N*-dimethylalkylamine. The detailed synthesis procedure and all analytical data were reported as Ref. [6].

Stock solutions of all fluorescent probes were prepared by dissolving the probes in DMSO and then diluting with Hepes buffer (15 mmol/L, pH 7.4) to obtain final concentration of 1 μmol/L (final DMSO concentration was <1% in the DOPC SUV experiment). DOPC SUV was prepared according to the published article [7]. Fluorescence spectra were recorded on a Hitachi F4500 spectrophotometer. Both N* and T* bands of the spectra were fitted by Gaussian functions. UV–vis absorption spectra were performed on a Shimadzu UV2550 UV–vis spectrophotometer. All the solvents were spectroscopic grade.

2. Results and discussion

F4N1 has been shown to be ultra sensitive to polarity and H-bonding capability of its microenvironment and has been used to detect dipole potential changes in biological membranes [1]. When bound to liposomes or plasma membranes of cells, it is located in the head group region of the phospholipid with the positively charged amine group exposed to aqueous phase [1]. Fourteen new derivatives of 3HFs have been synthesized with the hope that they may provide improved properties of **F4N1** for detection of dipole potential in biological systems

Fig. 1 shows the fluorescence emission of **F4N10** as an example of the new probes in Hepes buffer (15 mmol/L, pH 7.4), acetonitrile, dichloromethane, and when bound to DOPC SUV in Hepes buffer.

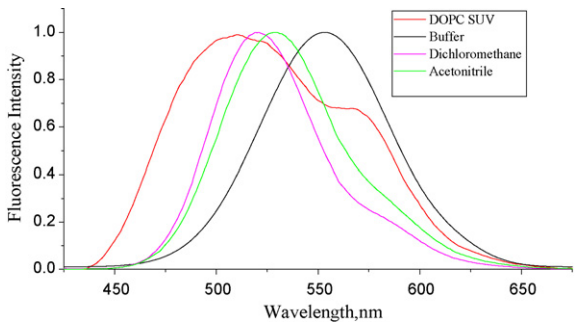

Fig. 1. Fluorescence emission of **F4N10** at concentration 1 μmol/L in Hepes buffer (15 mmol/L, pH 7.4) (Black), in dichloromethane (Purple), in acetonitrile (Green), and in DOPC SUV (Red) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

Table 1
Spectroscopic properties of the probes in different organic solvents.

Compound **5**			Acetonitrile					Dichloromethane				
	R_1	R_2	λ_{abs} (nm)	λ_{N^*} (nm)	λ_{T^*} (nm)	I_{N^*}/I_{T^*}	φ	λ_{abs} (nm)	λ_{N^*} (nm)	λ_{T^*} (nm)	I_{N^*}/I_{T^*}	φ
F2N1*	*n*-C_2H_5	CH_3	415	524	575	3.89	0.31	428	515	563	3.74	0.86
F2N4*	*n*-C_2H_5	*n*-C_4H_9	417	524	575	3.86	0.33	431	518	563	4.02	0.89
F2N6*	*n*-C_2H_5	*n*-C_6H_{13}	417	524	575	3.81	0.33	430	516	564	3.92	0.84
F2N7*	*n*-C_2H_5	*n*-C_7H_{15}	418	523	571	3.32	0.33	431	516	564	3.92	0.86
F2N8	*n*-C_2H_5	*n*-C_8H_{17}	417	524	576	3.89	0.32	430	517	564	3.95	0.87
F2N9*	*n*-C_2H_5	*n*-C_9H_{19}	415	524	575	3.79	0.32	429	517	565	3.89	0.89
F2N10*	*n*-C_2H_5	*n*-$C_{10}H_{21}$	418	524	575	3.86	0.31	430	517	565	4.06	0.88
F2N12*	*n*-C_2H_5	*n*-$C_{12}H_{25}$	418	524	577	4.02	0.34	430	517	566	4.20	0.88
F4N1	*n*-C_4H_9	CH_3	420	526	577	4.17	0.39	433	517	562	3.95	0.84
F4N4*	*n*-C_4H_9	*n*-C_4H_9	418	526	577	4.12	0.41	434	518	565	4.14	0.87
F4N6*	*n*-C_4H_9	*n*-C_6H_{13}	419	526	579	4.27	0.35	433	516	565	3.98	0.83
F4N7*	*n*-C_4H_9	*n*-C_7H_{15}	419	526	579	4.25	0.34	433	518	566	4.16	0.86
F4N8*	*n*-C_4H_9	*n*-C_8H_{17}	417	525	578	4.20	0.30	431	517	568	4.19	0.82
F4N9*	*n*-C_4H_9	*n*-C_9H_{19}	419	526	578	4.15	0.37	433	517	566	3.98	0.85
F4N10*	*n*-C_4H_9	*n*-$C_{10}H_{21}$	419	526	579	4.23	0.37	433	518	566	4.19	0.83
F4N12*	*n*-C_4H_9	*n*-$C_{12}H_{25}$	420	526	578	4.18	0.35	433	518	566	4.17	0.87

λ_{abs}, position of absorption maxima; $\lambda_{N^*}$and λ_{T^*}, position of fluorescence maxima of N* and T* forms, λ_{N^*}, λ_{T^*} and I_{N^*}/I_{T^*} are obtained from peakfit; φ is the fluorescence quantum yield using F2N8 in acetonitrile as the reference ($\varphi = 0.32$) [3]; excitation at 410 nm; *, new compound.

In Hepes buffer (15 mmol/L, pH 7.4), the fluorescence emission peak is found at 547 nm whose N* and I* bands are not resolved well. Relative to that in Hepes buffer, the emission peak is blue shifted to 526 nm and 517 nm in acetonitrile and dichloromethane, respectively; which also is accompanied by significantly increase in fluorescence quantum yield. The blue shift in dichloromethane is more than in acetonitrile, due to higher apolarity of dichloromethane. When bound to DOPC SUV, the emission peak also demonstrates blue shift with significantly increased fluorescence quantum yield. More importantly, the new probe showed very pronounced increase in T* band emission as indicated by the much decreased I_{N^*}/I_{T^*} value of 1.46 (Table 2).

Table 1 summarizes the spectroscopic properties of all the probes in acetonitrile and dichloromethane; Table 2 summarizes the spectroscopic properties of all probes in aqueous Hepes buffer (15 mmol/L, pH 7.4) and when bound to DOPC SUV. All data show that they have maximum absorption and emission shifted to shorter wavelengths with

Table 2
Spectroscopic properties of the probes in Hepes buffer and DOPC SUV.

Probe	Hepes buffer (15 mmol/L, pH 7.4)		DOPC SUV			
	λ_{abs} (nm)	λ_{N^*} (nm)	λ_{ex} (nm)	λ_{N^*} (nm)	λ_{T^*} (nm)	I_{N^*}/I_{T^*}
F2N1	429	550	423	489	548	0.89
F2N4	430	549	423	494	547	0.68
F2N6	431	550	424	499	552	0.84
F2N7	434	551	424	499	549	0.96
F2N8	436	551	424	501	554	0.96
F2N9	433	553	424	502	555	0.99
F2N10	432	552	425	501	553	0.93
F2N12	441	552	425	504	558	1.14
F4N1	436	554	426	492	546	1.12
F4N4	437	553	427	496	553	1.24
F4N6	440	556	427	499	559	1.4
F4N7	443	552	427	501	561	1.40
F4N8	448	554	428	501	562	1.40
F4N9	450	553	428	502	562	1.44
F4N10	449	547	427	502	563	1.46
F4N12	452	547	428	503	563	1.46

λ_{ex}, position of excited spectroscopic maxima recorded at 500 nm; λ_{N^*} and λ_{T^*}, position of fluorescence maxima of N* and T* forms; excitation wavelength 400 nm; λ_{N^*}, λ_{T^*} and I_{N^*}/I_{T^*} are obtained from peakfit.

significantly increased fluorescence quantum yield. Titration of all probes with DOPC SUV has indicated a drastic increase in quantum yields (date not shown).

Increase of the length of aliphatic chain at 6-position of 3HFs from one carbon to 12 carbons results in a gradual red shift of both excitation and emission maxima in both F2 and F4 series, which is accompanied by increased intensity ratio of N* and T* band, I_{N*}/I_{T*} (Table 2). This suggests that as the aliphatic chain length increases, the force to pull the 3HF moiety into the membranes from the positively charged side is increased; since the membrane is a strong barrier for cations to enter, the 3HF moiety has to adopt a different conformation in e.g. **F4N12** than in **F4N1** and the net result is that the 3HF moiety is buried shallower in the phospholipid head group region. This should allow 3HF moiety in for example **F4N12** to detect changes of dipole potential in the membrane at a different position and depth in the membrane, and this possibility is being explored.

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[6] Synthesis procedures: Compound **3** was prepared using a modification of Algar-Flynn-Ayamada reaction: to the solution of compound **1** (35 mmol) and compound **2** (35 mmol) (R_1 = *n*-C_2H_5, or *n*-C_4H_9) in EtOH (40 mL), 15 mL of aqueous solution of NaOH (10 g, 250 mmol) was added, the mixture was stirred at r.t. for overnight. To the resultant dark red solution, 10 mL of NaOH aqueous solution (2.5 g, 62.5 mmol) was added; the temperature was lowered to below 15 °C, 18 mL of 30% H_2O_2 solution was added. Then the temperature was raised to 40–60 °C and stirred for 0.5 h. After the reaction, pH was adjusted to 7 with diluted H_2SO_4 and extracted with ethyl acetate. The extracted compound **3** was concentrated with vacuum, and purified with silica gel to yield a bright yellow solid compound **3**. Compound **3** in 40% HBr solution (10 mL) was refluxed for 3 h, then the mixture was neutralized with $NaHCO_3$ and the precipitate was filtered to give compound **4**, which was used directly without further purification. Compound **5** were prepared as following: compound **4** (0.60 mmol) and *N,N*-dimethylalkylamine (0.70 mmol) was dissolved in ethanol (10 mL) and refluxed 8 h. After removal of solvents, the solid product was recrystallized with CH_2Cl_2 and ether to get compound **5**. Using the similar procedures, 16 derivatives of 3HFs were prepared and reported in Table 1. The melting points were determined on a RY-IG melting point apparatus and were uncorrected. ^{1}HNMR spectra were recorded on a Varian 600 MHz spectrometer using tetramethylsilane as an internal standard. HPLC–MS were recorded on Agilent 1100 Series LC/MSD. Selected analytical data for probes (compounds **5**): **F2N4**: yield: 69%, m.p. 180–181 °C, ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.00 (t, 3H, J = 7.2 Hz), 1.23 (t, 6H, J = 7.2 Hz), 1.45 (m, 2H), 1.85 (m, 2H), 3.34 (s, 6H), 3.46 (m, 4H), 3.62 (t, 2H, J = 8.4 Hz), 5.25 (s, 2H), 6.74 (d, 2H, J = 9 Hz), 6.92 (br, 1H), 7.58 (d, 1H, J = 9 Hz), 8.11 (d, 2H, J = 9 Hz), 8.22 (d, 1H, J = 1.2 Hz), 8.30 (dd, 1H, J = 8.4 Hz, J = 1.8 Hz), LC-MS: *m/z* 423 [M-Br^-]$^+$, ($C_{26}H_{35}BrN_2O_3$, 502). **F2N6**: yield: 71%, m.p. 145–147 °C, ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.87 (t, 3H, J = 7.2 Hz), 1.23 (t, 6H, J = 7.2 Hz), 1.28–1.38 (m, 6H), 1.84 (m, 2H), 3.33 (s, 6H), 3.44 (m, 4H), 3.59 (t, 2H, J = 9 Hz), 5.25 (s, 2H), 6.73 (d, 2H, J = 9.6 Hz), 6.97 (br, 1H), 7.55 (d, 1H, J = 9 Hz), 8.10 (d, 2H, J = 9 Hz), 8.23 (s, 1H), 8.28 (dd, 1H, J = 9 Hz, J = 1.8 Hz), LC-MS: *m/z* 451 [M-Br^-]$^+$, ($C_{28}H_{39}BrN_2O_3$, 530). **F2N8**: yield: 63%, m.p. 154–156 °C, ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.86 (t, 3H, J = 6.9 Hz), 1.22–1.83 (m, 18H), 3.33 (s, 6H), 3.45 (q, 4H, J = 7.2 Hz), 3.59 (t, 2H, J = 8.4 Hz), 5.26 (s, 2H), 6.74 (d, 2H, J = 9 Hz), 6.9 (br, 1H), 7.60 (d, 1H, J = 9 Hz), 8.12 (d, 2H, J = 9 Hz), 8.22 (d, 1H, J = 1.8 Hz), 8.33 (dd, 1H, J = 9 Hz, J = 1.8 Hz). **F2N10**: yield: 74%, m.p. 169–171 °C, ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.86 (t, 3H, J = 7.2 Hz), 1.22–1.84 (m, 22H), 3.33 (s, 6H), 3.45 (q, 4H, J = 7.2 Hz), 3.60 (t, 2H, J = 8.4 Hz), 5.25 (s, 2H), 6.73 (d, 2H, J = 9 Hz), 6.93 (br, 1H), 7.58 (d, 1H, J = 9 Hz), 8.11 (d, 2H, J = 9 Hz), 8.22 (d, 1H, J = 1.8 Hz), 8.31 (dd, 1H, J = 9 Hz, J = 1.8 Hz). **F2N12**: yield: 76%, m.p. 157–159 °C, ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.87 (t, 3H, J = 6.9 Hz), 1.22–1.82 (m, 28H), 3.32 (s, 6H), 3.45 (q, 4H, J = 7 Hz), 3.58 (t, 2H, J = 8.1 Hz), 5.24 (s, 2H), 6.74 (d, 2H, J = 9 Hz), 7.58 (d, 1H, J = 8.4 Hz), 8.11 (d, 2H, J = 9 Hz), 8.22 (s, 1H), 8.32 (d, 2H, J = 7.8 Hz), LC-MS: *m/z* 535 [M-Br^-]$^+$, ($C_{34}H_{51}BrN_2O_3$, 614). **F4N1**: yield: 58%, ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.92-0.95 (t, 6H, J = 7.2 Hz), 1.33–1.37 (m, 4H), 1.52–1.57 (m, 4H), 3.07 (s, 9H), 3.35–3.38 (t, 4H, J = 7.8 Hz), 4.70 (s, 2H), 6.79–6.80 (d, 2H, J = 9 Hz), 7.86 (t, 2H), 8.10–8.12 (d, 2H, J = 9 Hz), 8.29 (s, 1H), 9.35 (s, 1H), MS: *m/z*437 [M-Br^-]$^+$, ($C_{33}H_{49}BrN_2O_3$, 516). **F4N6**: yield: 59%, m.p. 164–166 °C, ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.87 (t, 3H, J = 6.9 Hz), 0.98 (t, 6H, J = 7.2 Hz), 1.29–1.44 (m, 10H), 1.61 (m, 4H), 1.83 (m, 2H), 3.34 (s, 6H), 3.35 (m, 4H), 3.58 (t, 2H, J = 8.4 Hz), 5.23 (s, 2H), 6.71 (d, 2H, J = 9 Hz), 6.91 (br, 1H), 7.58 (d, 1H, J = 9 Hz), 8.11 (d, 2H, J = 9 Hz), 8.23 (s, 1H), 8.31 (d, 1H, J = 8.4 Hz), LC-MS: *m/z* 507 [M-Br^-]$^+$, ($C_{32}H_{47}BrN_2O_3$, 586). **F4N10**: yield: 69%, m.p. 157–159 °C, ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.87 (t, 3H, J = 6.9 Hz), 0.99 (t, 6H, J = 7.2 Hz), 1.23–1.84 (m, 24H), 3.36 (m, 10H), 3.60 (t, 2H, J = 8.4 Hz), 5.26 (s, 2H), 6.71 (d, 2H, J = 9 Hz), 6.88 (br, 1H), 7.60 (d, 1H, J = 8.4 Hz), 8.12 (d, 2H, J = 9 Hz), 8.22 (s, 1H), 8.34 (d, 1H, J = 8.4 Hz), LC-MS: *m/z* 563 [M-Br^-]$^+$, ($C_{36}H_{55}BrN_2O_3$, 642). **F4N12**: yield: 72%, m.p. 166–168 °C, ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.87 (t, 3H, J = 7.2 Hz), 0.99 (t, 6H, J = 7.2 Hz), 1.23–1.42 (m, 22H), 1.62 (m, 4H), 1.83 (m, 2H), 3.34–3.37 (m, 10H), 3.60 (t, 2H, J = 8.4 Hz), 5.26 (s, 2H), 6.73 (d, 2H, J = 9 Hz), 6.83 (s, 1H), 7.62 (d, 1H, J = 8.4 Hz), 8.13 (d, 2H, J = 8.4 Hz), 8.20 (s, 1H), 8.38 (dd, 1H, J = 8.4 Hz, J = 1.8 Hz). Analytical data for other probes, **F2N1**, **F2N7**, **F2N9**, **F4N4**, **F4N7**, **F4N8** and **F4N9** are also available by request.
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