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Synthesis and activity evaluation of tilorone analogs as potential anticancer agents

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ABSTRACT

Tilorone is an interferon inducer with anticancer activity. Twenty-two novel tilorone analogs were synthesized by improvements of fluorenone skeleton, side chains and amino groups to screen new anticancer agents. *In vitro* evaluation showed that ten new compounds had better anticancer activities than tilorone. Among them, **2c** ($IC_{50} < 7$ μM against cancer cell lines and $IC_{50} > 35$ μM against non-cancer cell lines) and **5d** ($IC_{50} < 10$ μM against cancer cell lines and $IC_{50} > 53$ μM against non-cancer cell lines) exhibited the best anticancer activities and selectivities. Pharmacophore modeling of highly active compounds was carried out by Molecular Operating Environment (MOE) to generate a visualized model for compound design in future study.

1. Introduction

Tilorone (or 2,7-bis[2-diethylaminoethoxy]fluoren-9-one, Fig. 1) is the first synthetic, small molecular weight interferon inducer in possession of a broad range of therapeutical functions, such as resistance to systemic candidiasis, antiviral effect and anticancer activity [1–6]. These activities are mainly achieved by DNA strand intercalation that alters DNA chemobiological properties and further activates interferon expression [7–9].

Many previous studies showed that the modification of tilorone's fluorenone skeleton and side chain (Fig. 1) enhanced anticancer activity [10–16]. In general, the fluorenone skeleton was optimized by substitution with differently rigid planar rings [9,13,17–20], while the modification of the side chain was mostly derived from length extension, addition of carbonyl group and shift of linkage site [10,11,14,16] as well as change of *N*-alkyl groups [10–20]. It is thus of considerable interest to investigate the necessities of rigid skeleton and tertiary amino groups as well as the influence of extra hydroxyl groups.

On the basis of these considerations, twenty-two novel tilorone analogs containing rotational isoflavone skeleton with anticancer activity [21–24], hydroxyl side chains and non-tertiary amino groups have been designed, synthesized and assayed for cytotoxicity and anticancer activity. After *in vitro* activity evaluation, the highly active compounds were implemented pharmacophore modeling by Molecular Operating Environment (MOE) [25].

2. Chemistry

The compounds **1d** (Scheme 1) and **6c**–**m** (Scheme 2) were synthesized via a three-step procedure starting from daidzein (D) and 2,7-dihydroxy-9-fluorenone (F), respectively. This procedure included hydroxyethylation of phenolic hydroxyls by ethylene chlorohydrin, further chlorination of alcoholic hydroxyls by thionyl chloride and final nucleophilic substitution of chlorides by corresponding amines [26]. Note that in the first step, ethylene chlorohydrin in excess needed to be used due to its hydrolyzation during the reaction. The compounds **4c** and **4d** (Scheme 2) were produced via a similar procedure to **6c**–**m**, where the only difference was ethylation on one of the two phenolic hydroxyls by diethylsulfate prior to the three-step procedure. The synthesis of the compounds

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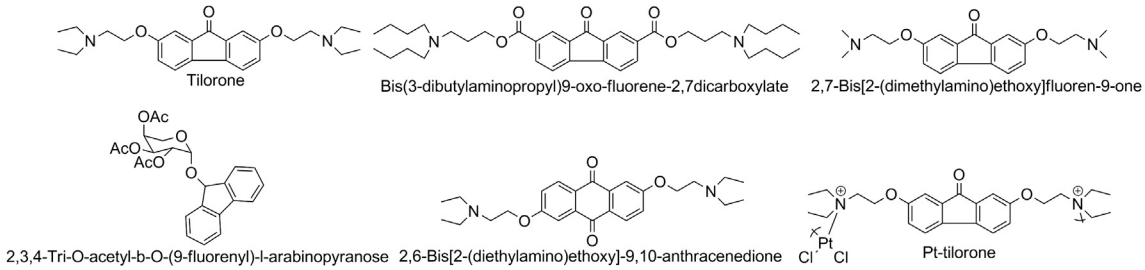
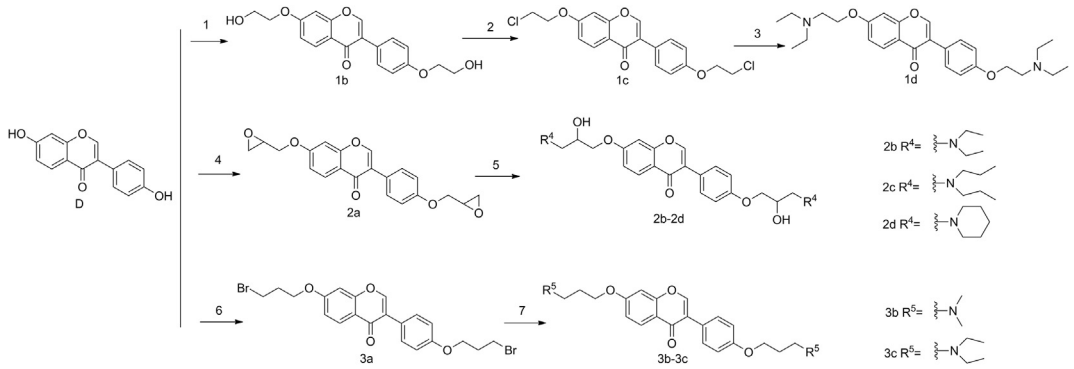
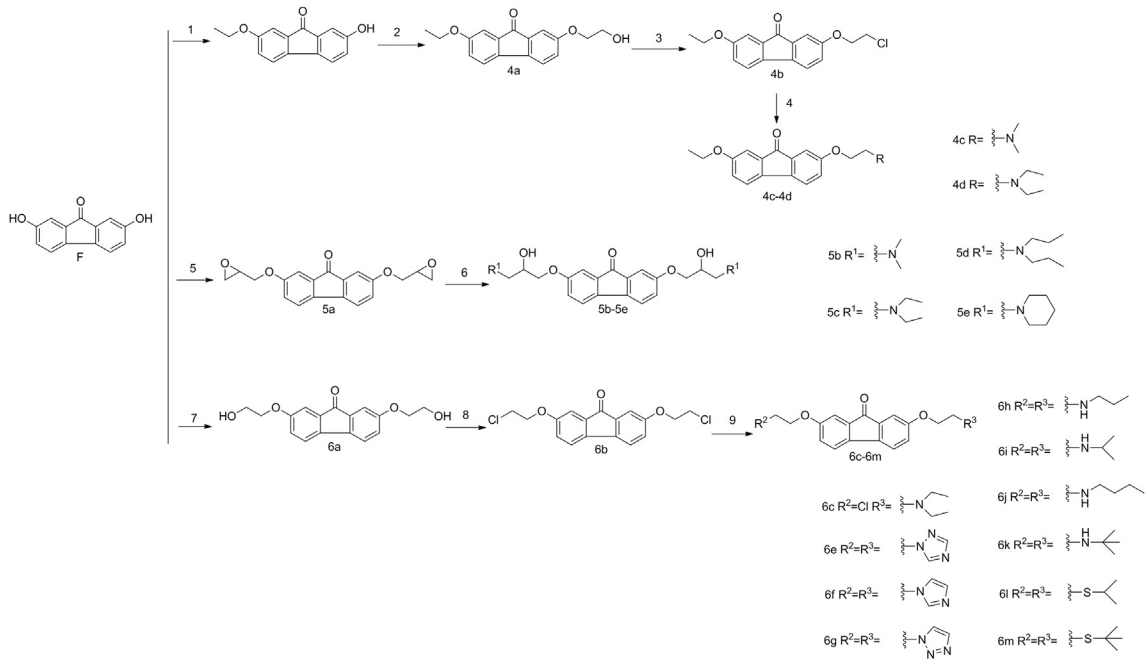


Fig. 1. Structures of tilorone and its active derivatives.



Scheme 1. Synthesis of compounds **1d**, **2b**–**d**, **3b** and **3c**. Reagents and conditions: (1) NaOH/ethylene chlorohydrin/DMSO, 70 °C; (2) thionyl chloride, 60 °C; (3) potassium carbonate/diethylamine/$CHCl_3$, reflux; (4) epichlorohydrin/NaOH/DMSO, 70 °C; (5) potassium carbonate/amines/$CHCl_3$, reflux; (6) NaOH/1,3-dibromopropane/DMSO, 70 °C; (7) potassium carbonate/amines/DMF, 70 °C.

2b–**d** (Scheme 1) and **5b**–**e** (Scheme 2) involved epoxypropylation of starting compounds (D and F) by epichlorohydrin followed by ring-opening reaction of epoxide using appropriate amines. The compounds **3b** and **3c** (Scheme 1) were obtained by bromopropylation and nucleophilic substitution. Bromopropylation was performed with an excess of 1,3-dibromopropane (five equivalents) in order to reduce by-products. Our attempts indicated that direct bromoethylation by 1,2-dibromoethane could not be used to prepare **1d** and **6c**–**m**, resulted in a large amount of polymolecular by-products.



Scheme 2. Synthesis of compounds **4c**, **4d**, **5b**–**e** and **6c**–**m**. Reagents and conditions: (1) NaOH/diethylsulfate/water, R.T.; (2) NaOH/ethylene chlorohydrin/DMSO, 70 °C; (3) thionyl chloride, 60 °C; (4) potassium carbonate/amines/DMF, 70 °C; (5) epichlorohydrin/NaOH/DMSO, 70 °C; (6) potassium carbonate/amines/DMF, 70 °C; (7) NaOH/ethylene chlorohydrin/DMSO, 70 °C; (8) thionyl chloride, 60 °C; (9) potassium carbonate/amines/DMF, 70 °C.

Table 1
Anticancer and cytotoxic activities of synthesized compounds.

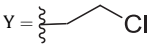
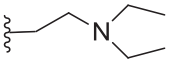
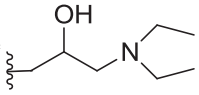
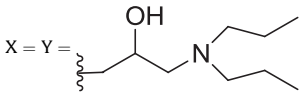
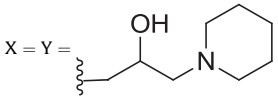
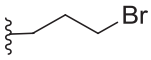
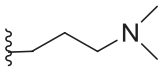
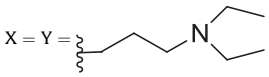
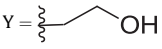
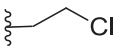
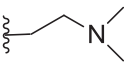
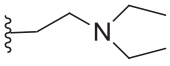
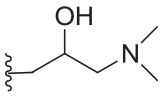
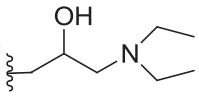
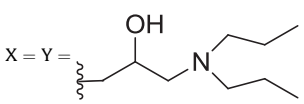
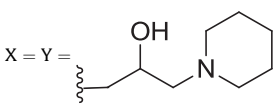
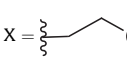
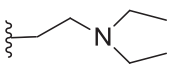
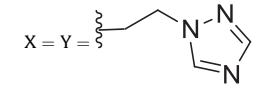
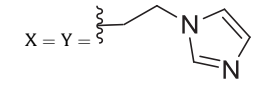
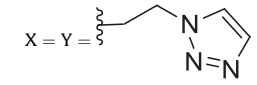
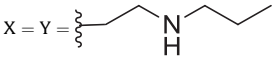
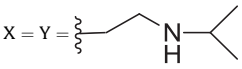
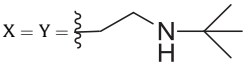
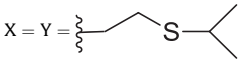
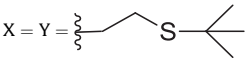
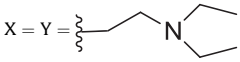
Compounds	X, Y	IC_{50}[a] (μM)				
		Cancer cell lines			Non-cancer cell lines	
		Hep3B	HeLa	A549	Vero	293T
1c	X = Y = 	N. A.[b]	N. A.	N. A.	N. D.[c]	N. D.
1d	X = Y = 	35.22	26.39	19.43	102.41	106.68
2b	X = Y = 	30.36	27.84	24.98	60.11	52.87
2c	X = Y = 	6.52	5.17	4.26	35.48	39.65
2d	X = Y = 	12.00	5.32	4.34	18.53	20.16
3a	X = Y = 	N. A.	N. A.	N. A.	N. D.	N. D.
3b	X = Y = 	24.37	22.18	20.63	34.92	41.89
3c	X = Y = 	76.14	82.37	73.51	N. D.	N. D.
4a	X = CH_2CH_3, Y = 	N. A.	N. A.	N. A.	N. D.	N. D.
4b	X = CH_2CH_3, Y = 	N. A.	N. A.	N. A.	N. D.	N. D.
4c	X = CH_2CH_3, Y = 	>100	>100	>100	N. D.	N. D.
4d	X = CH_2CH_3, Y = 	84.75	106.63	>100	N. D.	N. D.
5b	X = Y = 	18.31	11.73	12.58	20.95	28.26
5c	X = Y = 	21.46	20.23	16.04	48.57	56.38
5d	X = Y = 	9.43	6.78	7.42	57.83	53.97
5e	X = Y = 	6.76	1.82	1.40	13.01	17.95
6c	X =  Y = 	>100	>100	>100	N. D.	N. D.

Table 1 (*continued*)

Compounds	X, Y	IC_{50}[a] (μM)				
		Cancer cell lines			Non-cancer cell lines	
		Hep3B	HeLa	A549	Vero	293T
6e		>100	>100	>100	N. D.	N. D.
6f		>100	>100	>100	N. D.	N. D.
6g		>100	>100	>100	N. D.	N. D.
6h		5.17	2.73	2.96	8.47	8.59
6i		8.89	1.72	2.43	14.76	13.81
6j		3.34	2.21	2.65	8.89	10.97
6k		20.08	9.17	7.86	25.33	19.82
6l		N. A.	N. A.	N. A.	N. D.	N. D.
6m		N. A.	N. A.	N. A.	N. D.	N. D.
Tilorone		38.78	39.97	32.63	89.53	76.41

Compounds **1c**, **1d**, **2b**–**d** and **3a**–**c** are daidzein skeleton.
Compounds **4a**–**d**, **5b**–**e** and **6c**–**m** are fluorenone skeleton.
[a] IC_{50} is the half maximal (50%) cell growth inhibitory concentration of the compound.
[b] N. A. means no activity at concentration of 100 μM or higher.
[c] N. D. means not determined.

The structures of the synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, elemental analysis and mass spectra. The data are shown in the Experimental part.

3. Results and discussion

3.1. In vitro anticancer activity

The *in vitro* anticancer activities of the synthesized compounds were evaluated according to MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay by using tilorone as positive control. Three human cancer cell lines, human hepatoma cell line (Hep3B), human cervical cancer cell line (HeLa) and human lung adenocarcinoma epithelial cell line (A549), were used for screening. The anticancer activities are described by half maximal inhibitory concentration (IC_{50}) values in Table 1.

There are ten new compounds (**2c**, **2d**, **3b**, **5b**–**e** and **6i**–**k**) which showed strong inhibition ($IC_{50} < 25$ μM) against cancer cell lines. The positive control tilorone's IC_{50} was 32–39 μM to cancer cell lines. These new compounds with **1d**, **2b**, **6h** and tilorone were then tested against two non-cancer cell lines, African green monkey kidney epithelial cells (Vero) and human embryonic kidney cells (293T), for investigating their cytotoxicities. The IC_{50} values are summarized in Table 1.

The results of anticancer and cytotoxic assay reveal that the compounds without amino group (**1c**, **3a**, **4a**, **4b**, **6l** and **6m**) or possessing only one amino group (**4c**, **4d** and **6c**) were not actively against cancer cells even though the concentrations were higher than 100 μM. This result shows double amino groups are critical for anticancer activities. When the amino groups are substituted by hydroxyls, halogens, mercapto groups or there was only one amino group, the compounds lose cytotoxicity. Compounds **6e**, **6f** and **6g** with two azoles instead of amino groups exhibited low activities ($IC_{50} > 100$ μM). The reason probably is the azole has weaker ability of forming hydrogen bond than amine and the rigid ring is not benefit to activity.

The compounds **6h**–**k** with two secondary amino groups performed much better activities to cancer cell lines (3.34–20.08 μM against Hep3B, 1.72–9.17 μM against HeLa and 2.43–7.86 μM against A549) than tilorone. However, they exhibited high cytotoxicities to non-cancer cell lines (8.47–25.33 μM against Vero and 8.59–19.82 μM against 293T, tilorone was 89.53 μM against Vero and 76.41 μM against 293T). The differences of activity between **6h**–**k** and tilorone might derive from toxic secondary metabolites hydroxylamines. Although this class of compounds is effectively against cancer cells, they have high cytotoxicities.

Compounds **5b**–**e** possess all the functional groups as tilorone with an extra hydroxyl on each side chain. All of them exhibited

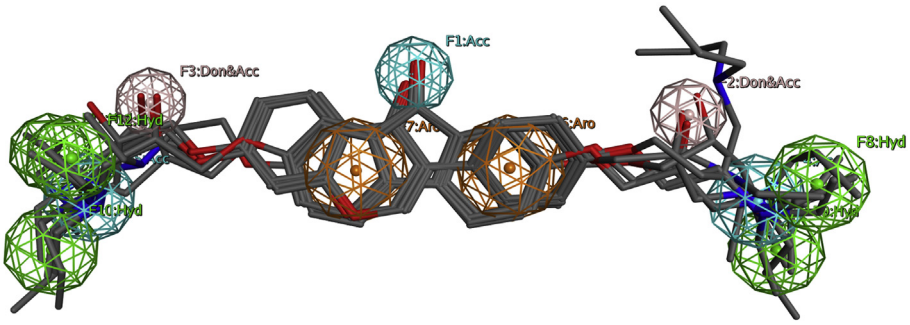


Fig. 2. Ligand-based pharmacophore model. The pharmacophore features were generated by assembled pharmacophore annotation points. Pharmacophore features: cyan, H-bond acceptor; pink, H-bond donor and/or acceptor; brown, aromatic centroid; green, hydrophobic centroid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

better activities than tilorone. **5d** (9.43 μM against Hep3B, 6.78 μM against HeLa and 7.42 μM against A549) and **5e** (6.76 μM against Hep3B, 1.82 μM against HeLa and 1.40 μM against A549) were an order of magnitude more active than tilorone. The inhibition to non-cancer cell lines showed **5e** was cytotoxic to tested cell lines (13.01 μM against Vero and 17.95 μM against 293T), but **5d** exhibited good selectivity between cancer and non-cancer cells (57.83 μM against Vero and 53.97 μM against 293T). This class of compounds indicates that extra hydroxyl on side chain increases anticancer activity and flexible carbon chain of tertiary amino group is in favor of selectivity.

The compounds **1d**, **2b**–**d**, **3b** and **3c** were synthesized to determine the necessity of fluorenone skeleton for anticancer activity. The isoflavone skeleton has a rotational aromatic ring other than rigid skeleton. The IC_{50} values of **1d** against cancer cell lines and non-cancer cell lines were slightly lower than tilorone. **3b** and **3c** are side chain extension compounds of **1d**. There is somewhat deficiency for their activities. The most active compounds with isoflavone skeleton are those with hydroxyls on their side chains such as **2c** and **2d**. **2c** performed excellent anticancer activity (6.52 μM against Hep3B, 5.17 μM against HeLa and 4.26 μM against A549) and was good at selectivity (35.48 μM against Vero and 39.65 μM against 293T). This result implies that the totally rigid skeleton is not necessary for anticancer activity.

3.2. Pharmacophore modeling

The results of *in vitro* anticancer activity assay have proposed some structure–activity relationships about active compounds. In this part, we intended to use molecular simulation to build a model for guiding compound design in the future. Since the target DNA segment of tilorone has not been ascertained yet [7,8,27,28], the ligand-based drug design has to be employed in the near future. As a significant tool of ligand-based drug design, pharmacophore modeling can generate a visualized spatial model of active compounds which could be used for directing compound design and virtual screening. Hence, we set up a pharmacophore model from the highly active tilorone analogs (**1d**, **2b**, **2c**, **3b** and **5b**–**d**) and tilorone (Fig. 2). The procedure included flexible alignment of the active compounds and pharmacophore consensus [29].

This pharmacophore model suggests that the compounds derived from fluorenone and isoflavone share similar pharmacophore features. The carbonyl groups as well as the tertiary amino groups locate at the same site, while the benzene rings form hydrophobic centers. The pharmacophore model also shows that the pharmacophores of synthesized compounds superpose and fit tilorone's pharmacophores well. Thus the model presents the probable pharmacophores spatial arrangements and can help us to design compounds in future study.

4. Conclusions

Twenty-two novel tilorone analogs were synthesized and screened as candidates of novel anticancer agents. There are ten new compounds that performed better anticancer activities than tilorone. **2c** and **5d** are the most potent compounds for both anticancer activities and selectivities. A brief sum-up of structure–activity relationship is outlined as follows: 1. amino groups are necessary for the anticancer activity; 2. comparing with tertiary amino groups, secondary amino groups increase the anticancer activity but decrease the selectivity; 3. the introduced hydroxyls on side chains improve the activity considerably; 4. the substitution of fluorenone skeleton by isoflavone skeleton retains the anticancer activity. Pharmacophore model shows the visualized spatial arrangements of pharmacophores and can be used to direct compound design.

5. Experimental protocols

5.1. Chemistry

Melting points were determined on a XT-4 apparatus (uncorrected) and some of the compounds were not determined due to hygroscopicity or oil like. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 apparatus in $CDCl_3$ or DMSO-d_6 with TMS as internal standard. Chemical shifts were given in δ (ppm) and coupling constants (J) were shown in Hz. Mass spectra of all new compounds were recorded by electrospray ionization (ESI) method on a LC/MSD Trap SL Plus spectrometer (Agilent Technologies, Waldbronn, Germany). Optical rotations were measured on a PerkinElmer Model 341 polarimeter. Elemental analysis was performed on a VarioEL III instrument. The reactions were monitored by thin layer chromatography (TLC).

5.1.1. 4′,7-Bis(2-hydroxyethoxy)isoflavone (*1b*)

Daidzein (10 g, 0.0394 mol) was solved in 150 ml DMSO, NaOH (3.2 g, 0.08 mol) and ethylene chlorohydrin (6.7 ml, 0.1 mol) were added into the solution. The resulting solution was stirred overnight at 70 °C, and then the solution was poured into 1 M NaOH water solution. The precipitate was filtered and washed by 1 M NaOH till colorless eluate and dried in vacuum drying oven then recrystallized from 2-propanol to obtain compound **1b**. Light yellow solid. Yield 51%. M.p. 189–191 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.43 (s, 1H), 8.03 (d, J = 8.9 Hz, 1H), 7.56–7.49 (m, 2H), 7.18 (d, J = 2.3 Hz, 1H), 7.10 (dd, J = 8.9, 2.4 Hz, 1H), 7.00 (d, J = 8.8 Hz, 2H),

4.15 (t, J = 4.8 Hz, 2H), 4.02 (t, J = 5.0 Hz, 2H), 3.75 (dt, J = 14.6, 4.9 Hz, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ: 174.62, 163.14, 158.42, 157.39, 153.44, 130.05, 126.91, 123.98, 123.33, 117.49, 115.08, 114.13, 101.03, 70.46, 69.48, 59.42, 59.18. Anal. Calcd. for $C_{19}H_{18}O_6$: C 66.66, H 5.30; Found C 66.58, H 5.31.

*5.1.2. 4′,7-Bis(2-chloroethoxy)isoflavone (**1c**)*

1b (2 g, 0.00585 mol) was solved in 40 ml thionyl chloride and stirred at 60 °C till the reaction completed (monitored by TLC), then poured the solution into 1 M NaOH carefully. The precipitate was collected and purified by flash column chromatography to yield compound **1c**. Light yellow solid. Yield 40%. M.p. 166–168 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.15 (d, J = 8.9 Hz, 1H), 7.85 (s, 1H), 7.43 (d, J = 8.7 Hz, 2H), 6.98–6.87 (m, 3H), 6.80 (d, J = 2.2 Hz, 1H), 4.23 (dt, J = 25.2, 5.8 Hz, 4H), 3.78 (dt, J = 17.1, 5.8 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 175.74, 162.45, 158.21, 157.78, 152.23, 130.26, 128.09, 124.91, 124.83, 118.89, 114.80, 114.63, 101.07, 68.42, 68.10, 41.85, 41.43. Anal. Calcd. for $C_{19}H_{16}Cl_2O_4$: C 60.17, H 4.25; Found C 60.20, H 4.25. MS (ESI): m/z $[M + H]^+$ calcd 379.0, found 378.9.

*5.1.3. 4′,7-Bis(2-diethylaminoethoxy)isoflavone (**1d**)*

To a stirred solution of **1c** (0.4 g, 0.00106 mol) in 50 ml $CHCl_3$, potassium carbonate (0.5 g, 0.0036 mol) and diethylamine (1 ml, 0.01 mol) were added. The reaction was refluxed 24 h and extracted with brine and CH_2Cl_2. The organic layer was filtered, washed with brine and dried. After concentrated in vacuum, the crude product was separated on flash column to obtain compound **1d**. Light yellow solid. Yield 75%. M.p. 80–81 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.09 (d, J = 8.9 Hz, 1H), 7.83 (s, 1H), 7.40 (d, J = 8.6 Hz, 2H), 6.89 (t, J = 8.9 Hz, 3H), 6.78 (d, J = 1.9 Hz, 1H), 4.03 (dt, J = 17.6, 6.1 Hz, 4H), 2.83 (q, J = 6.0 Hz, 4H), 2.58 (q, J = 7.1 Hz, 8H), 1.01 (t, J = 7.1 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 175.83, 163.23, 158.77, 157.85, 152.11, 130.06, 127.54, 124.71, 124.21, 118.26, 114.96, 114.48, 100.65, 67.36, 66.43, 51.62, 51.41, 47.87, 47.73, 11.81, 11.73. Anal. Calcd. for $C_{27}H_{36}N_2O_4$: C 71.65, H 8.02 N 6.19; Found C 71.59, H 8.04, N 6.18.

*5.1.4. 4′,7-Bis(2,3-epoxypropoxy)isoflavone (**2a**)*

Daidzein (10 g, 0.0394 mol) was solved in 150 ml DMSO, NaOH (3.2 g, 0.08 mol) and epichlorohydrin (16 ml, 0.2 mol) were added into the solution. The solution was stirred overnight at 70 °C. The resulting solution was then poured into 1 M NaOH water solution. The precipitate was filtered and washed by 1 M NaOH till colorless eluate and dried in vacuum drying oven to obtain crude product. The crude product was separated on flash column to get compound **2a**. Light yellow solid. Yield 55%. M.p. 117–118 °C. $[\alpha]_D^{20}$ 0° (c 0.5, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, J = 8.9 Hz, 1H), 7.85 (s, 1H), 7.42 (t, J = 5.8 Hz, 2H), 6.95 (dd, J = 9.0, 2.4 Hz, 1H), 6.92 (d, J = 8.8 Hz, 2H), 6.82 (d, J = 2.3 Hz, 1H), 4.33–4.16 (m, 2H), 3.94 (td, J = 11.1, 5.8 Hz, 2H), 3.37–3.28 (m, 2H), 2.87 (dt, J = 15.0, 4.5 Hz, 2H), 2.72 (ddd, J = 10.6, 4.8, 2.6 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.78, 162.70, 158.49, 157.79, 152.21, 130.19, 127.97, 124.82, 124.75, 118.79, 114.73, 114.68, 101.06, 69.31, 68.81, 50.15, 49.79, 44.76, 44.58. Anal. Calcd. for $C_{21}H_{18}O_6$: C 68.85, H 4.95; Found C 68.81, H 4.96.

*5.1.5. General procedure for the synthesis of compounds **2b–d***

To a stirred solution of **2a** (0.4 g, 0.00109 mol) in 50 ml $CHCl_3$, potassium carbonate (0.5 g, 0.0036 mol) and diethylamine (1 ml, 0.01 mol) were added. The reaction was refluxed 24 h and extracted with brine and CH_2Cl_2. The organic layer was filtered, washed with brine and dried. After concentrated, the crude product was separated on flash column to obtain compound **2b**. Compounds **2c** and **2d** were produced by reacted with dipropylamine and piperidine respectively in the same method.

*5.1.5.1. 4′,7-Bis(3-diethylamino-2-hydroxypropoxy)isoflavone (**2b**).* Light yellow solid. Yield 80%. M.p. 87–88 °C. $[\alpha]_D^{20}$ 0° (c 0.5, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.13 (d, J = 8.9 Hz, 1H), 7.85 (s, 1H), 7.42 (d, J = 8.7 Hz, 2H), 6.98–6.88 (m, 3H), 6.82 (d, J = 2.3 Hz, 1H), 4.05–3.88 (m, 6H), 2.70–2.50 (m, 12H), 1.02 (td, J = 7.1, 5.0 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 175.85, 163.13, 158.76, 157.84, 152.15, 130.13, 127.79, 124.83, 124.51, 118.57, 114.84, 114.59, 100.86, 70.99, 70.41, 65.73, 65.56, 56.14, 55.73, 47.38, 47.28, 11.83, 11.65. Anal. Calcd. for $C_{29}H_{40}N_2O_6$: C 67.94, H 7.86 N 5.46; Found C 67.89, H 7.87, N 5.45.

*5.1.5.2. 4′,7-Bis(3-dipropylamino-2-hydroxypropoxy)isoflavone (**2c**).* Yellow oil. Yield 65%. $[\alpha]_D^{20}$ 0° (c 0.5, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.20 (d, J = 8.9 Hz, 1H), 7.92 (s, 1H), 7.49 (d, J = 8.7 Hz, 2H), 7.07–6.96 (m, 3H), 6.89 (d, J = 2.3 Hz, 1H), 4.10–3.97 (m, 6H), 2.64–2.40 (m, 12H), 1.59–1.41 (m, 8H), 0.90 (td, J = 7.3, 2.5 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 175.86, 163.18, 158.86, 157.84, 152.13, 130.10, 127.78, 124.84, 124.43, 118.55, 114.84, 114.60, 100.82, 71.02, 70.55, 65.92, 65.70, 57.23, 56.93, 56.25, 56.22, 20.30, 20.29, 11.80. Anal. Calcd. for $C_{33}H_{48}N_2O_6$: C 69.69, H 8.51 N 4.93; Found C 69.65, H 8.52, N 4.92. MS (ESI): m/z $[M + H]^+$ calcd 569.4, found 569.1.

*5.1.5.3. 4′,7-Bis(3-piperidino-2-hydroxypropoxy)isoflavone (**2d**).* Light yellow solid. Yield 85%. M.p. 161–163 °C. $[\alpha]_D^{20}$ 0° (c 0.5, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.20 (d, J = 8.9 Hz, 1H), 7.92 (s, 1H), 7.49 (d, J = 8.7 Hz, 2H), 7.05–6.94 (m, 3H), 6.89 (d, J = 2.2 Hz, 1H), 4.16 (dd, J = 12.5, 5.7 Hz, 2H), 4.09–3.96 (m, 4H), 2.73–2.42 (m, 12H), 1.64 (d, J = 4.4 Hz, 8H), 1.48 (t, J = 11.9 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 175.85, 163.09, 158.71, 157.83, 152.17, 130.14, 127.80, 124.81, 124.53, 118.57, 114.85, 114.60, 100.85, 70.93, 70.34, 65.14, 64.98, 61.31, 60.92, 54.78, 25.88, 25.69, 24.04, 23.94. Anal. Calcd. for $C_{31}H_{40}N_2O_6$: C 69.38, H 7.51 N 5.22; Found C 69.35, H 7.52, N 5.21. MS (ESI): m/z $[M + H]^+$ calcd 537.3, found 537.1.

*5.1.6. 4′,7-Bis(3-bromopropoxy)isoflavone (**3a**)*

Daidzein (5 g, 0.0197 mol) was solved in 100 ml DMSO, NaOH (1.6 g, 0.04 mol) and 1,3-dibromopropane (10 ml, 0.1 mol) were added into the solution. The resulting solution was stirred overnight at 70 °C. The solution was then poured into 1 M NaOH water solution. The precipitate was filtered and washed by 1 M NaOH till colorless eluate and dried in vacuum drying oven to obtain crude product. The crude product was separated on flash column to obtain compound **3a**. Light yellow solid. Yield 70%. M.p. 138–141 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.22 (d, J = 8.9 Hz, 1H), 7.92 (d, J = 2.8 Hz, 1H), 7.53–7.46 (m, 2H), 7.02–6.95 (m, 3H), 6.88 (d, J = 2.3 Hz, 1H), 4.19 (dt, J = 29.9, 5.8 Hz, 4H), 3.63 (dd, J = 11.4, 6.3 Hz, 4H), 2.43–2.29 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 175.85, 163.00, 158.69, 157.90, 152.12, 130.20, 127.90, 124.89, 124.48, 118.59, 114.76, 114.59, 100.76, 65.94, 65.37, 32.33, 31.98, 30.02, 29.59. Anal. Calcd. for $C_{21}H_{20}Br_2O_4$: C 50.83, H 4.06; Found C 50.78, H 4.05. MS (ESI): m/z $[M - Br]^+$ calcd 415.1, found 414.9.

*5.1.7. General procedure for the synthesis of compounds **3b** and **3c***

To a stirred solution of **3a** (0.5 g, 0.001 mol) in 40 ml dimethylformamide, potassium carbonate (0.5 g, 0.0036 mol) and dimethylamine solution (33%, 3.1 ml, 0.02 mol) were added. The reaction was taken 24 h at 70 °C and then extracted with brine and CH_2Cl_2. The organic layer was filtered, washed with brine and dried. After concentrated in vacuum, the crude product was separated on flash column to obtain compound **3b**. **3c** was prepared by instead of dimethylamine solution with diethylamine in the same condition.

*5.1.7.1. 4′,7-Bis(3-dimethylaminopropoxy)isoflavone (**3b**).* Light yellow solid. Yield 75%. Hygroscopicity. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.12 (d, J = 8.9 Hz, 1H), 7.84 (s, 1H), 7.44–7.37 (m, 2H), 6.88 (d,

J = 8.6 Hz, 3H), 6.78 (d, J = 2.3 Hz, 1H), 4.05 (t, J = 6.3 Hz, 2H), 3.98 (t, J = 6.3 Hz, 2H), 2.54 (t, J = 7.5 Hz, 2H), 2.49 (t, J = 7.5 Hz, 2H), 2.29 (s, 6H), 2.26 (s, 6H), 1.97 (dd, J = 13.7, 6.8 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 175.88, 163.31, 158.85, 157.93, 152.10, 130.13, 127.70, 124.81, 124.28, 118.33, 114.86, 114.49, 100.62, 66.72, 66.01, 56.13, 55.97, 45.18, 44.93, 26.92. Anal. Calcd. for $C_{25}H_{32}N_2O_4$: C 70.73, H 7.60, N 6.60; Found C 70.70, H 7.61, N 6.58. MS (ESI): m/z $[M + H]^+$ calcd 425.2, found 425.0.

*5.1.7.2. 4′,7-Bis(3-diethylaminopropoxy)isoflavone (**3c**).* Light yellow solid. Yield 70%. M.p. 82–84 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.19 (d, J = 8.9 Hz, 1H), 7.92 (s, 1H), 7.49 (d, J = 8.6 Hz, 2H), 6.97 (dd, J = 15.5, 5.4 Hz, 3H), 6.86 (d, J = 2.0 Hz, 1H), 4.12 (dt, J = 26.9, 5.9 Hz, 4H), 2.95–2.74 (m, 12H), 2.25–2.08 (m, 4H), 1.25 (t, J = 7.2 Hz, 6H), 1.17 (t, J = 7.2 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 175.87, 163.18, 158.60, 157.93, 152.16, 130.20, 127.75, 124.79, 124.53, 118.41, 114.85, 114.47, 100.65, 66.57, 65.63, 49.32, 49.18, 46.93, 46.86, 30.95, 25.89, 25.36, 10.77, 10.13. Anal. Calcd. for $C_{29}H_{40}N_2O_4$: C 72.47, H 8.39, N 5.83; Found C 72.45, H 8.40, N 5.83. MS (ESI): m/z $[M + H]^+$ calcd 481.3, found 481.1.

*5.1.8. 2-(2-Hydroxyethoxy)-7-ethoxyfluoren-9-one (**4a**)*

2,7-Dihydroxy-fluoren-9-one (10 g, 0.047 mol) was added into 200 ml water with NaOH (4 g, 0.1 mol), then stirred in room temperature till completely solved. Diethylsulfate (9.2 ml, 0.07 mol) was added dropwise into the system. The mixture was stirred 5 h and the precipitate was filtered out and dried. The precipitate was then solved in 80 ml DMSO, NaOH (2 g, 0.05 mol) and ethylene chlorohydrin (4.1 ml, 0.06 mol) were added. The reaction was carried out overnight at 70 °C, then the solution was poured into 1 M NaOH and precipitate was collected by filtration. After dried in vacuum oven, the mixture was stirred in 50 ml CH_2Cl_2 for half an hour, then filtered the insoluble substance and washed with CH_2Cl_2 then recrystallized from 2-propanol to get **4a**. Orange solid. Yield 30%. M.p. 158–160 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.27 (s, 1H), 7.25 (s, 1H), 7.13 (dd, J = 4.4, 2.4 Hz, 2H), 6.93 (ddd, J = 10.4, 8.2, 2.4 Hz, 2H), 4.12–4.08 (m, 2H), 4.05 (q, J = 7.0 Hz, 2H), 3.98 (s, 2H), 1.42 (t, J = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.73, 159.41, 158.94, 137.97, 137.22, 135.99, 135.92, 120.93, 120.81, 120.63, 120.55, 110.30, 110.18, 69.71, 64.00, 61.35, 14.76. Anal. Calcd. for $C_{17}H_{16}O_4$: C 71.82, H 5.67; Found C 71.79, H 5.69. MS (ESI): m/z $[M + H]^+$ calcd 285.1, found 285.0.

*5.1.9. 2-(2-Chloroethoxy)-7-ethoxyfluoren-9-one (**4b**)*

4a (2 g, 0.007 mol) was solved in 40 ml thionyl chloride and stirred at 60 °C till the reaction completed (monitored by TLC), and then poured the solution into 1 M NaOH carefully. The precipitate was collected and purified by flash column chromatography to yield compound **4b**. Orange solid. Yield 90%. M.p. 146.6–147.4 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.23 (d, J = 2.3 Hz, 1H), 7.21 (d, J = 2.3 Hz, 1H), 7.08 (d, J = 1.9 Hz, 2H), 6.88 (ddd, J = 14.3, 8.1, 2.5 Hz, 2H), 4.19 (t, J = 5.8 Hz, 2H), 3.99 (q, J = 7.0 Hz, 2H), 3.75 (t, J = 5.8 Hz, 2H), 1.35 (t, J = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 196.44, 159.50, 158.50, 138.30, 137.14, 136.03, 135.95, 121.19, 120.97, 120.70, 120.62, 110.32, 110.19, 68.48, 64.02, 41.76, 14.76. Anal. Calcd. for $C_{17}H_{15}ClO_3$: C 67.44, H 4.99; Found C 67.41, H 5.01. MS (ESI): m/z $[M - Cl]^+$ calcd 267.4, found 267.3.

*5.1.10. General procedure for the synthesis of compounds **4c** and **4d***

To a stirred solution of **4b** (0.4 g, 0.0013 mol) in 40 ml dimethylformamide, potassium carbonate (0.5 g, 0.0036 mol) and dimethylamine solution (33%, 1.5 ml, 0.01 mol) were added. The reaction was carried out in 24 h at 70 °C and then extracted with brine and CH_2Cl_2. The organic layer was filtered, washed with brine and dried. After concentrated, the crude product was separated on flash column to obtain compound **4c**. **4d** was prepared by instead of dimethylamine solution with diethylamine in the same condition.

*5.1.10.1. 2-(2-Dimethylaminoethoxy)-7-ethoxyfluoren-9-one (**4c**).* Orange solid. Yield 85%. M.p. 80–81.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.19 (d, J = 8.1 Hz, 2H), 7.06 (d, J = 7.0 Hz, 2H), 6.89 (d, J = 8.0 Hz, 1H), 6.84 (d, J = 8.0 Hz, 1H), 4.03 (t, J = 5.4 Hz, 2H), 3.98 (dd, J = 13.8, 6.9 Hz, 2H), 2.70 (t, J = 5.3 Hz, 2H), 2.30 (s, 6H), 1.34 (t, J = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.77, 159.35, 159.08, 137.72, 137.31, 135.95, 135.93, 120.99, 120.85, 120.56, 120.49, 110.19, 110.14, 66.31, 63.98, 58.11, 45.77, 14.76. Anal. Calcd. for $C_{19}H_{21}NO_3$: C 73.29, H 6.80 N 4.50; Found C 73.24, H 6.82, N 4.51. MS (ESI): m/z $[M + H]^+$ calcd 312.2, found 312.0.

*5.1.10.2. 2-(2-Diethylaminoethoxy)-7-ethoxyfluoren-9-one (**4d**).* Orange solid. Yield 88%. M.p. 60–61 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.21 (s, 1H), 7.18 (s, 1H), 7.07 (t, J = 2.4 Hz, 2H), 6.86 (dd, J = 7.9 Hz, 2H), 4.05 (t, J = 5.9 Hz, 2H), 3.98 (q, J = 6.9 Hz, 2H), 2.89 (t, J = 5.8 Hz, 2H), 2.66 (q, J = 7.1 Hz, 4H), 1.35 (t, J = 7.0 Hz, 3H), 1.05 (t, J = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.77, 159.36, 159.00, 137.68, 137.33, 135.98, 135.96, 120.91, 120.60, 120.58, 120.49, 110.49, 110.14, 66.56, 63.99, 51.48, 47.78, 14.76, 11.41. Anal. Calcd. for $C_{21}H_{25}NO_3$: C 74.31, H 7.42 N 4.13; Found C 74.26, H 7.43, N 4.12.

*5.1.11. 2,7-Bis(2,3-epoxypropoxy)fluoren-9-one (**5a**)*

2,7-Dihydroxy-fluoren-9-one (10 g, 0.047 mol) was solved in 150 ml DMSO, NaOH (4 g, 0.1 mol) and epichlorohydrin (16 ml, 0.2 mol) were added into the solution. The solution was stirred overnight at 70 °C. The resulting solution was then poured into 1 M NaOH water solution. The precipitate was collected and washed by 1 M NaOH till colorless eluate and dried in vacuum drying oven to obtain crude product. The crude product was separated on flash column to get compound **5a**. Orange solid. Yield 73%. M.p. 93–95 °C. $[\alpha]_D^{20}$ 0° (c 0.5, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.31 (d, J = 8.2 Hz, 2H), 7.16 (d, J = 2.2 Hz, 2H), 6.99 (dd, J = 8.2, 2.3 Hz, 2H), 4.29 (dd, J = 11.0, 2.8 Hz, 2H), 3.96 (dd, J = 11.0, 5.8 Hz, 2H), 3.37 (d, J = 2.8 Hz, 2H), 2.93 (t, J = 4.5 Hz, 2H), 2.78 (dd, J = 4.7, 2.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.37, 158.93, 137.87, 135.97, 121.08, 120.72, 110.33, 69.21, 49.99, 44.57. Anal. Calcd. for $C_{19}H_{16}O_5$: C 70.36, H 4.97; Found C 70.33, H 5.00.

*5.1.12. General procedure for the synthesis of compounds **5b–e***

To a stirred solution of **5a** (0.4 g, 0.00123 mol) in 50 ml dimethylformamide, potassium carbonate (0.5 g, 0.0036 mol) and diethylamine (1 ml, 0.01 mol) were added. The reaction was processed 24 h at 70 °C and extracted with brine and CH_2Cl_2. The organic layer was filtered, washed with brine and dried. After concentrated in vacuum, the crude product was separated on flash column to obtain compound **5c**. Compounds **5b**, **5d** and **5e** were produced by reacted with dimethylamine solution, dipropylamine and piperidine respectively in the same method.

*5.1.12.1. 2,7-Bis(3-dimethylamino-2-hydroxypropoxy)fluoren-9-one (**5b**).* Orange solid. Yield 75%. M.p. 95–97 °C. $[\alpha]_D^{20}$ 0° (c 0.5, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.18 (d, J = 8.1 Hz, 2H), 7.07 (d, J = 2.3 Hz, 2H), 6.89 (dd, J = 8.2, 2.4 Hz, 2H), 4.03–3.96 (m, 2H), 3.91 (qd, J = 9.6, 4.8 Hz, 4H), 2.48 (dd, J = 12.1, 9.9 Hz, 2H), 2.29 (dd, J = 12.3, 3.7 Hz, 2H), 2.25 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 192.57, 158.14, 136.62, 134.87, 119.85, 119.56, 109.26, 69.90, 65.14, 60.63, 44.58. Anal. Calcd. for $C_{23}H_{30}N_2O_5$: C 66.65, H 7.30, N 6.76; Found C 66.63, H 7.32, N 6.75. MS (ESI): m/z $[M + H]^+$ calcd 415.2, found 415.0.

*5.1.12.2. 2,7-Bis(3-diethylamino-2-hydroxypropoxy)fluoren-9-one (**5c**).* Orange solid. Yield 80%. M.p. 103–105 °C. $[\alpha]_D^{20}$ 0° (c 0.5,

CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.31 (d, J = 8.2 Hz, 1H), 7.14 (d, J = 2.3 Hz, 1H), 6.99 (dd, J = 8.2, 2.4 Hz, 1H), 4.19 (dd, J = 9.0, 4.4 Hz, 1H), 4.04 (dt, J = 9.6, 4.5 Hz, 2H), 2.93–2.77 (m, 6H), 1.18 (t, J = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.53, 159.01, 137.53, 135.75, 120.70, 120.66, 110.43, 70.71, 65.29, 55.18, 47.51, 11.06. Anal. Calcd. for $C_{27}H_{38}N_2O_5$: C 68.91, H 8.14, N 5.95; Found C 68.93, H 8.16, N 5.94. MS (ESI): m/z $[M + H]^+$ calcd 471.3, found 471.1.

*5.1.12.3. 2,7-Bis(3-dipropylamino-2-hydroxypropoxy)fluoren-9-one (**5d**).* Orange oil. Yield 80%. $[\alpha]_D^{20}$ 0° (c 0.5, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.15 (d, J = 8.1 Hz, 2H), 7.03 (d, J = 2.0 Hz, 2H), 6.86 (dd, J = 8.1, 2.1 Hz, 2H), 4.00 (td, J = 9.5, 4.8 Hz, 2H), 3.91 (d, J = 4.8 Hz, 2H), 2.60–2.39 (m, 12H), 1.54–1.37 (m, 8H), 0.83 (t, J = 7.3 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.54, 159.11, 137.58, 135.84, 120.69, 120.58, 110.35, 70.78, 65.73, 56.96, 56.14, 19.82, 11.70. Anal. Calcd. for $C_{31}H_{46}N_2O_5$: C 70.69, H 8.80, N 5.32; Found C 70.64, H 8.81, N 5.33. MS (ESI): m/z $[M + H]^+$ calcd 527.3, found 527.1.

*5.1.12.4. 2,7-Bis(3-piperidino-2-hydroxypropoxy)fluoren-9-one (**5e**).* Orange solid. Yield 85%. M.p. 126–128 °C. $[\alpha]_D^{20}$ 0° (c 0.5, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.22 (d, J = 8.1 Hz, 2H), 7.12 (d, J = 2.3 Hz, 2H), 6.94 (dd, J = 8.2, 2.4 Hz, 2H), 4.08 (td, J = 9.3, 4.7 Hz, 2H), 4.02–3.92 (m, 4H), 2.60 (d, J = 4.3 Hz, 4H), 2.52–2.32 (m, 8H), 1.67–1.52 (m, 8H), 1.44 (dd, J = 12.9, 6.1 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.52, 159.23, 137.60, 135.89, 120.86, 120.53, 110.35, 71.08, 65.37, 61.02, 54.75, 26.05, 24.20. Anal. Calcd. for $C_{29}H_{38}N_2O_5$: C 70.42, H 7.74, N 5.66; Found C 70.38, H 7.75, N 5.68. MS (ESI): m/z $[M + H]^+$ calcd 495.3, found 495.1.

*5.1.13. 2,7-Bis(2-hydroxyethoxy)fluoren-9-one (**6a**)*

2,7-Dihydroxy-fluoren-9-one (20 g, 0.094 mol) was solved in 200 ml DMSO, NaOH (8 g, 0.2 mol) and ethylene chlorohydrin (13.4 ml, 0.2 mol) were added into the solution. The resulting solution was stirred overnight at 70 °C, and then poured into 1 M NaOH water solution. The precipitate was washed by 1 M NaOH till colorless eluate and dried in vacuum drying oven then recrystallized from 2-propanol to obtain compound **6a**. Orange solid. Yield 55%. M.p. 166–168 °C. ^{1}H NMR (400 MHz, DMSO) δ: 7.54 (s, 2H), 7.08 (s, 4H), 4.05 (s, 4H), 3.72 (s, 4H). ^{13}C NMR (101 MHz, DMSO) δ: 192.81, 159.09, 136.72, 135.07, 121.41, 120.82, 110.06, 70.08, 59.45. Anal. Calcd. for $C_{17}H_{16}O_5$: C 67.99, H 5.37; Found C 68.01, H 5.37.

*5.1.14. 2,7-Bis(2-chloroethoxy)fluoren-9-one (**6b**)*

6a (10 g, 0.033 mol) was solved in 80 ml thionyl chloride and stirred at 60 °C till the reaction completed (monitored by TLC), then poured the solution into 1 M NaOH carefully. The precipitate was collected and purified by flash column chromatography to yield compound **6b**. Orange solid. Yield 90%. M.p. 142–144 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.29 (d, J = 8.1 Hz, 2H), 7.14 (d, J = 2.3 Hz, 2H), 6.97 (dd, J = 8.1, 2.4 Hz, 2H), 4.25 (t, J = 5.8 Hz, 4H), 3.82 (t, J = 5.8 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.25, 158.68, 137.98, 136.00, 121.23, 120.81, 110.39, 68.49, 41.75. Anal. Calcd. for $C_{17}H_{14}Cl_2O_3$: C 60.55, H 4.18; Found C 60.53, H 4.20.

*5.1.15. General procedure for the synthesis of compounds **6c**–**m***

To a stirred solution of **6b** (0.4 g, 0.0012 mol) in 40 ml dimethylformamide, potassium carbonate (0.5 g, 0.0036 mol) and diethylamine (1 ml, 0.01 mol) were added. The reaction was taken 24 h at 70 °C and then extracted with brine and CH_2Cl_2. The organic layer was filtered, washed with brine and dried. After concentrated in vacuum, the crude product was separated on flash column to obtain compound **6c**. Compounds **6e**–**k** were prepared by instead of diethylamine by according amines in the same condition. **6l** and **6m** required NaH as base and anhydrous dimethylformamide with isopropyl mercaptan and *t*-butyl mercaptan, respectively.

*5.1.15.1. 2-(2-Chloroethoxy)-7-(2-diethylaminoethoxy)fluoren-9-one (**6c**).* Orange solid. Yield 40%. M.p. 75–76.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.19 (dd, J = 8.1, 1.8 Hz, 2H), 7.06 (dd, J = 5.1, 2.4 Hz, 2H), 6.87 (ddd, J = 8.0, 5.4, 2.4 Hz, 2H), 4.17 (t, J = 5.8 Hz, 2H), 4.02 (t, J = 6.0 Hz, 2H), 3.74 (t, J = 5.8 Hz, 2H), 2.85 (t, J = 6.0 Hz, 2H), 2.62 (q, J = 7.1 Hz, 4H), 1.03 (t, J = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.45, 159.28, 158.52, 138.17, 137.29, 135.99, 135.91, 121.14, 120.72, 120.67, 110.48, 110.31, 68.46, 66.79, 51.52, 47.79, 41.77, 11.57. Anal. Calcd. for $C_{21}H_{24}ClNO_3$: C 67.46, H 6.47, N 3.75; Found C 67.44, H 6.46, N 3.75. MS (ESI): m/z $[M + H]^+$ calcd 374.1, found 374.0.

*5.1.15.2. 2,7-Bis[2-(1H-1,2,4-triazol-1-yl)ethoxy]fluoren-9-one (**6e**).* Orange solid. Yield 55%. M.p. 209–212 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.22 (s, 2H), 7.97 (s, 2H), 7.28 (d, J = 8.2 Hz, 2H), 7.11 (d, J = 2.4 Hz, 2H), 6.89 (dd, J = 8.2, 2.5 Hz, 2H), 4.59 (t, J = 5.0 Hz, 4H), 4.36 (t, J = 5.0 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 192.99, 160.26, 158.33, 152.24, 144.01, 138.05, 135.97, 120.91, 110.28, 66.08, 49.12. Anal. Calcd. for $C_{21}H_{18}N_6O_3$: C 62.68, H 4.51, N 20.88; Found C 62.67, H 4.53, N 20.87. MS (ESI): m/z $[M + H]^+$ calcd 403.1, found 403.0.

*5.1.15.3. 2,7-Bis[2-(1H-imidazol-1-yl)ethoxy]fluoren-9-one (**6f**).* Orange solid. Yield 50%. M.p. 128–130 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.63 (s, 2H), 7.28 (d, J = 1.2 Hz, 2H), 7.09 (m, 6H), 6.91 (dd, J = 8.2, 2.4 Hz, 2H), 4.36 (t, J = 4.9 Hz, 4H), 4.24 (t, J = 5.0 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.14, 158.42, 138.01, 137.56, 135.93, 129.60, 121.00, 120.92, 119.38, 110.14, 67.68, 45.79. Anal. Calcd. for $C_{23}H_{20}N_4O_3$: C 68.99, H 5.03, N 13.99; Found C 68.98, H 5.05, N 13.96. MS (ESI): m/z $[M + H]^+$ calcd 401.2, found 401.0.

*5.1.15.4. 2,7-Bis[2-(1H-1,2,3-triazol-1-yl)ethoxy]fluoren-9-one (**6g**).* Orange solid. Yield 50%. M.p. 186–189 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.87 (dd, J = 6.6, 3.0 Hz, 2H), 7.39 (dd, J = 6.6, 3.0 Hz, 2H), 7.22 (d, J = 8.2 Hz, 2H), 7.13 (d, J = 2.2 Hz, 2H), 6.90 (dd, J = 8.2, 2.3 Hz, 2H), 5.11 (t, J = 5.5 Hz, 4H), 4.68 (t, J = 5.5 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.11, 158.50, 137.97, 135.90, 126.57, 121.22, 120.71, 118.09, 110.45, 66.43, 55.49. Anal. Calcd. for $C_{21}H_{18}N_6O_3$: C 62.68, H 4.51, N 20.88; Found C 62.65, H 4.53, N 20.89. MS (ESI): m/z $[M + H]^+$ calcd 403.1, found 402.9.

*5.1.15.5. 2,7-Bis(2-propylaminoethoxy)fluoren-9-one (**6h**).* Orange solid. Yield 80%. Hygroscopicity. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.26 (d, J = 8.1 Hz, 1H), 7.14 (d, J = 2.4 Hz, 1H), 6.93 (dd, J = 8.2, 2.5 Hz, 1H), 4.09 (t, J = 5.2 Hz, 2H), 3.01 (t, J = 5.1 Hz, 2H), 2.68–2.62 (m, 2H), 2.34 (s, 1H), 1.55 (dd, J = 14.7, 7.4 Hz, 2H), 0.94 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.65, 159.25, 137.53, 135.93, 120.66, 120.54, 110.33, 67.96, 51.76, 48.64, 23.21, 11.77. Anal. Calcd. for $C_{23}H_{30}N_2O_3$: C 72.22, H 7.91, N 7.32; Found C 72.20, H 7.93, N 7.32.

*5.1.15.6. 2,7-Bis(2-isopropylaminoethoxy)fluoren-9-one (**6i**).* Orange solid. Yield 82%. Hygroscopicity. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.18 (d, J = 8.1 Hz, 1H), 7.06 (s, 1H), 6.86 (d, J = 7.9 Hz, 1H), 4.01 (t, J = 4.8 Hz, 2H), 2.92 (t, J = 4.8 Hz, 2H), 2.80 (dt, J = 12.3, 6.1 Hz, 1H), 1.61 (s, 1H), 1.03 (d, J = 6.2 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 192.61, 158.22, 136.51, 134.91, 119.61, 119.50, 109.35, 67.21, 47.52, 45.23, 21.92. Anal. Calcd. for $C_{23}H_{30}N_2O_3$: C 72.22, H 7.91, N 7.32; Found C 72.18, H 7.94, N 7.30. MS (ESI): m/z $[M + H]^+$ calcd 383.2, found 383.1.

*5.1.15.7. 2,7-Bis(2-butylaminoethoxy)fluoren-9-one (**6j**).* Orange solid. Yield 75%. Hygroscopicity. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.19 (d, J = 8.1 Hz, 1H), 7.07 (d, J = 2.2 Hz, 1H), 6.86 (dd, J = 8.1, 2.4 Hz, 1H), 4.02 (t, J = 5.1 Hz, 2H), 2.93 (t, J = 5.1 Hz, 2H), 2.64–2.57 (m, 2H), 1.70 (s, 1H), 1.46–1.39 (m, 2H), 1.34–1.24 (m, 2H), 0.86 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.69, 159.25, 137.54, 135.93, 120.67, 120.55, 110.33, 67.94, 49.62, 48.72, 32.24, 20.47, 14.04. Anal. Calcd. for

$C_{25}H_{34}N_2O_3$: C 73.14, H 8.35, N 6.82; Found C 73.11, H 8.37, N 6.81. MS (ESI): m/z $[M + H]^+$ calcd 411.3, found 411.1.

*5.1.15.8. 2,7-Bis(2-tert-butylaminoethoxy)fluoren-9-one (**6k**).* Orange solid. Yield 80%. Hygroscopicity. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.10 (d, J = 8.1 Hz, 1H), 6.94 (d, J = 1.9 Hz, 1H), 6.81–6.72 (m, 1H), 3.93 (s, 2H), 2.78 (s, 2H), 0.98 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.55, 159.06, 137.31, 135.69, 120.47, 120.42, 110.35, 63.21, 52.85, 8.06. Anal. Calcd. for $C_{25}H_{34}N_2O_3$: C 73.14, H 8.35, N 6.82; Found C 73.13, H 8.36, N 6.82. MS (ESI): m/z $[M + H]^+$ calcd 411.3, found 411.0.

*5.1.15.9. 2,7-Bis(2-isopropylthioethoxy)fluoren-9-one (**6l**).* Orange solid. Yield 50%. M.p. 85.2–86.8 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.20 (d, J = 8.3 Hz, 2H), 7.07 (s, 2H), 6.86 (d, J = 6.4 Hz, 2H), 4.07 (t, J = 6.8 Hz, 4H), 2.98 (dt, J = 13.3, 6.6 Hz, 2H), 2.85 (t, J = 6.8 Hz, 4H), 1.24 (d, J = 6.7 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.56, 158.96, 137.67, 135.99, 120.86, 120.62, 110.37, 68.53, 35.41, 29.38, 23.49. Anal. Calcd. for $C_{23}H_{28}O_3S_2$: C 66.31, H 6.77; Found C 66.28, H 6.77. MS (ESI): m/z $[M + H]^+$ calcd 416.2, found 416.8.

*5.1.15.10. 2,7-Bis(2-tert-butylthioethoxy)fluoren-9-one (**6m**).* Orange solid. Yield 54%. M.p. 105.2–106.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.19 (d, J = 8.0 Hz, 1H), 7.07 (d, J = 1.8 Hz, 1H), 6.86 (dd, J = 8.1, 2.0 Hz, 1H), 4.05 (t, J = 7.1 Hz, 2H), 2.84 (t, J = 7.1 Hz, 2H), 1.29 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 193.53, 158.96, 137.67, 136.00, 120.89, 120.59, 110.40, 68.43, 42.47, 31.06, 27.53. Anal. Calcd. for $C_{25}H_{32}O_3S_2$: C 67.53, H 7.25; Found C 67.51, H 7.26. MS (ESI): m/z $[M + H]^+$ calcd 445.2, found 445.1.

5.2. Evaluation of in vitro anticancer activity

The evaluation cells (1×10^4 cells/well) were inoculated to standard 96-well microtiter plates in 100 μl of Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and incubated overnight at 37 °C in a humidified atmosphere containing 5% CO_2 incubator. The compounds were diluted to five different concentrations (1 μM–100 μM) in DMEM and added 100 μl to each well. After 48 h incubation, 10 μl of 5 mg/ml MTT was added to each well and the microtiter plates were incubated another 4 h at 37 °C in the incubator [30]. Then the supernatant in each well was taken out carefully and 100 μl of DMSO was added into each well to dissolve the formazon crystal. The absorbance at 490 nm wavelength was measured by a microplate spectrophotometer. Each assay was parallelly performed at least three times and the IC_{50} values were determined by regression analysis.

5.3. Pharmacophore modeling

Eight compounds (**1d**, **2b**, **2c**, **3b**, **5b–d** and tilorone) were chosen for generating ligand-based pharmacophore model by MOE. The partial charges and hydrogens of the compounds were preliminarily adjusted, and then the compounds were flexibly aligned under forcefield MMFF94x. The best alignment was selected to undertake pharmacophore consensus with default parameters.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.03.050.

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