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Construction of the oxaphenalene skeletons of mansonone F derivatives through C–H bond functionalization and their evaluation for anti-proliferative activities[†]

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Novel mansonone F derivatives were conveniently synthesized *via* a key step of Ru(II)-catalyzed C-H functionalization to rapidly construct oxaphenalene skeletons. This synthetic procedure is sufficiently robust and flexible to offer both the generation of diverse mansonone F analogs and the scale-up synthesis of selected compounds. The structural formulas of all products were confirmed and characterized using spectral data. Most of the derivatives exhibited significant cytotoxicity against four tested human tumor cell lines *in vitro*.

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Introduction

Natural products have long been an important source of drugs or drug leads. Natural products or their derivatives still comprise more than 50% of the drugs that are used for cancer chemotherapy.1 Many natural quinones and quinoid heterocycles have been reported to show antitumor effects. For example, tanshinone IIa,^{2,3} crytotanshinone, dunnione, β-lapachone,^{4,5} and mansonone E and F^{6,7} show great potential as antitumor agents (Fig. 1). Unlike the common o-quinone compounds with structural features of naphtho[1,2-b]furan or benzo[h]chromene, mansonone F (Fig. 1, MsF), a tricyclic sesquiterpenoid naturally occurring as ortho-naphthoquinone with an unusual oxaphelane skeleton, was first isolated from the heartwood of a west African tree Mansonia altissima A. Chev. (Sterculiaceae).8-10 MsF is a natural phytoalexin and MsF derivatives have attracted considerable attention because of their significant antimicrobial, antifungal, antioxidant, larvicidal and anticancer activities.11,12

To date, several approaches for synthesizing MsF and its derivatives have been reported. For instance, intramolecular Diels–Alder addition of benzynes to furans and intramolecular Friedel–Crafts acylation has been utilized for this purpose.¹³ Based on the regioselective hydrolysis of a hydroquinone diacetate and IBX (2-iodoxybenzoic acid) as oxidant for the MsF,¹⁴ the oxaphenalene skeleton of MsF has also been constructed by

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peri ring closure of naphthol ether.^{15,16} However, these multistep synthetic routes are generally time-consuming as they present inherent structural constraints that limit further optimization of their biological properties through structural modification. Furthermore, the existing routes are hampered by low efficiency in constructing the parent rings. Therefore, development of a new synthetic route for biologically active MsF derivatives is necessary. A novel synthetic route for modification *via* a key step of Ru(II)-catalyzed C–H functionalization has also been used to construct the oxaphenalene skeleton and shorten previously used synthetic routes.^{17,18} Oxidative C–H bond functionalization provides economical access to important bioactive heteroarenes and thus has been extensively used in medicinal chemistry.¹⁹

In this study, a series of highly active derivatives were successfully synthesized by establishing a novel and efficient



Fig. 1 The structures of natural o-quinone compounds.

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synthetic route for MsF derivatives. The method is convenient and a one-step reaction is sufficient to construct the key tricyclic oxaphenalene intermediate using $C(sp^2)$ –H activation through Ru(π)-catalyzed cyclization. The synthesized compounds were then evaluated for their anti-proliferative activities with different cancer cell lines *in vitro*.

Results and discussion

Chemistry

catalysts

17

18

19

 NO_2

 NO_2

н

The starting material, 1-naphthol, was treated with hex-3-yne in anhydrous dimethylformamide (DMF) with [RuCl₂(p-cymene)]₂ as a catalyst and $Cu(OAc)_2 \cdot H_2O$ to obtain the key tricyclic oxaphenalene intermediate (Table 1, entry 1). Furthermore, the reactivity of the o-substituted 1-naphthol was investigated. o-Chloro-1-naphthol has low yield of 30% when $(Cp*RhCl_2)_2$ was used as catalyst (Table 1, entry 9). We found that o-methyl, obromo or o-nitro naphthol could not form the oxaphenalene structure even under 150 °C and under the presence of RhCp*(CH₃CN)(SbF₆)₂ (Table 1, entries 2-7 and 10-18). Orthosubstituents have a large steric hindrance effect on the cyclization reactivity. In addition, the reaction yield of 4-nitro-1naphthol is higher than that of 1-naphthol (Table 1, entry 19). The reason is that the presence of electron-withdrawing groups leads to the stronger acidity of phenols and the easier dissociation of phenoxy anions. Thus, 4-nitro-1-naphthol was used as the starting substrate and $[RuCl_2(p-cymene)]_2$ as the catalyst to

Table 1 Scope of the oxidative cyclization reactions using Ru(II) as

prepare the oxaphenalene intermediates. Introduction of substituent groups is the optimal strategy to synthesize Msn derivatives. The reactivity of different alkynes was also investigated in this cyclization reaction. The result showed that only alkyl or aromatic alkynes can participate in the reaction (Table 2, entries 1–15). Aromatic substituted alkynes illustrate an easier reaction with higher yield and faster speed than that of alkyl alkynes (Table 2, entries 4–12). Interestingly, the terminal and TMS-protected alkynes could not afford the expected product in this catalytic system (Table 2, entries 22 and 23).

The proposed MsF derivatives were obtained as outlined in Scheme 1 and 2.²⁰ The key tricyclic oxaphenalene intermediate 2 was constructed through a one-step Ru(II)-catalyzed cyclization reaction based on the above optimized conditions. The two subsequent steps were performed according to a previously described method with minor modification.⁷ The nitro group of intermediate 2 was reduced to amine by using sodium hydrosulfite as the reducing reagent. The amine was oxidized by Fremy's salt, thereby generating target compounds 3 (Scheme 1).

MsF derivatives substituted at the C-9 position were obtained from 3a to 3f *via* different methods. Fluoro-substituted MsF derivatives 4 were obtained by using Selectfluor®. Meanwhile, chloro- and bromo-substituted MsF derivatives 5 and 6 were obtained by using *N*-chlorosuccinimide and *N*-bromosuccinimide,

Table 2 Reaction of 4-nitronaphthalene-1-ol with alkynes

 \mathbb{R}^2 Catalysts _____ Cu(OAc)₂· H₂O DMF. 16h \mathbf{R}^2 \mathbb{R}^1 Yield [%] Entry $T(^{\circ}C)$ Catalysts Н Н 100 $[RuCl_2(p-cymene)]_2$ 55 1 2 CH_3 Н 100 $[RuCl_2(p-cymene)]_2$ Trace 3 CH_3 Η 150 [RuCl₂(p-cymene)]₂ Trace 4 CH_3 Η 100 $(Cp*RhCl_2)_2$ Trace 5 CH₃ Н 150(Cp*RhCl₂)₂ Trace RhCp*(CH₃CN)(SbF₆)₂ 6 CH₃ н 100 Trace 7 CH₃ Н 150 RhCp*(CH₃CN)(SbF₆)₂ Trace 8 ClΗ 100 $[RuCl_2(p-cymene)]_2$ Trace 9 ClΗ 100 (Cp*RhCl₂)₂ 30 10 Br Η 100 [RuCl₂(p-cymene)]₂ Trace 11 Br H 150 [RuCl₂(p-cymene)]₂ Trace (Cp*RhCl₂)₂ 12 Br 100 Н Trace 13 Br Н 150 (Cp*RhCl₂)₂ Trace RhCp*(CH₃CN)(SbF₆)₂ 14 Br Η 100 Trace Н RhCp*(CH₃CN)(SbF₆)₂ 15 Br 150 Trace 16 NO_2 Η 100 $[RuCl_2(p-cymene)]_2$ Trace

(Cp*RhCl₂)₂

RhCp*(CH₃CN)(SbF₆)₂

 $[RuCl_2(p-cymene)]_2$

Trace

Trace

75

OH	+	_{B³} _{P4} [RuCl₂(p-cymene)]₂	NO ₂
NO ₂	+	$R^{\circ} = R^{\circ} R^{\circ} = DMF, Cu(OAc)_2 H_2O$	$R^3 \qquad \downarrow 0 \\ R^4 \qquad \qquad$

Entry	R ³	R^4	$T(^{\circ}C)$	Time (h)	Yield (%)
1	C_2H_5	C_2H_5	100	6	26
2	C_2H_5	C_2H_5	100	12	65
3	C_2H_5	C_2H_5	100	16	75
4	CH ₃	Ph	60	3	35
5	CH_3	Ph	60	9	58
6	CH_3	Ph	100	3	76
7	CH_3	Ph	100	9	88
8	CH_2CH_3	Ph	100	9	84
9	Ph	Ph	60	3	28
10	Ph	Ph	60	9	46
11	Ph	Ph	100	3	70
12	Ph	Ph	100	9	85
13	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	100	16	78
14	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	100	16	74
15	$(CH_2)_4CH_3$	$(CH_2)_4CH_3$	100	16	78
16	COOC ₂ H ₅	$COOC_2H_5$	100	16	0
17	CN	CN	100	16	0
18	CH_2OH	CH_2OH	100	16	0
19	CH_2Cl	CH_2Cl	100	16	0
20	COOH	COOH	100	16	0
21	COOH	Ph	100	16	0
22	Н	Ph	100	16	0
23	$Si(CH_3)_3$	Ph	100	16	0
24	CH_2Cl	Ph	100	16	0

н

Н

 NO_2

100

100

100



Scheme 1 Reagents and conditions: (i) alkyne, $[RuCl_2(p-cymene)]_2$, $Cu(OAc)_2 \cdot H_2O$, DMF, 80 °C, 14 h; (ii) $Na_2S_2O_4$, THF/H_2O, 50 °C,1 h; (iii) acetone, 0.06 M KH_2PO_4, Fremy's salt, 1 h; (iv) Selectfluor, MeCN, 70 °C, 4 h; (v) NCS, BPO, CCl_4, 80 °C,4 h; (vi) NBS, BPO, CCl_4, 80 °C, 4 h; (vii) Cu(NO_3)_2 \cdot 3H_2O, Ac₂O, 20 min.



respectively. Nitro-substituted MsF derivatives 7 were obtained by using cupric nitrate (Scheme 1).

Furthermore, 4,5-disubstituted MsF derivatives were synthesized from 6,7-difluoronaphthalen-1-ol **10**. However, given that it is not commercially available, the starting 1-bromo-2,4,5
 Table
 3
 Reaction
 of
 6,7-difluoronaphthalen-1-ol
 with

 diethylacetylene

 <



trifluorobenzene 8 was treated with Mg in anhydrous THF. Afterward, furan was added via Diels-Alder cyclization to obtain 6,7-difluoro-1,4-dihydro-1,4-epoxynaphthalene 9.21,22 The O-ring opening of 9 was created by adding BF3-etherate in dichloromethane obtaining 6,7-difluoronaphthalen-1-ol 10. For the compounds with 6,7-difluoro group, the yield in the C-H functional step was improved from 10% to 45% (Table 3) when mxylene was used as solvent instead of DMF. The 5-amination products 15 were produced by treating 12 with various amine and K₂CO₃ in DMF. Based on the information of NOESY spectra of compound 17, both 6-H(δ = 7.79 ppm) and piperidyl α -CH₂ (δ = 3.23 ppm) have a through-space correlation. Piperidyl should be considered only as a 5-position substituent group. This finding is possibly due to the C-5 position having lower steric hindrance and higher electron density than the C-4 position, which makes electrophilic substitution more likely at the C-5 position. As shown in Scheme 2, target compounds 13 and 16a-16d were obtained by using the strategy mentioned in Scheme 1, whereas 13 and 16a were brominated by using N-bromosuccinimide in carbon tetrachloride to obtain 14 and 17, respectively (Scheme 2). All structures of the novel intermediates and target compounds were confirmed by ¹H NMR, ¹³C NMR and HRMS (ESI).

Anti-proliferation activities and summary of SARs

Cytotoxicity of all the synthesized compounds was evaluated against four different human cancer cell lines. The inhibitory activities (IC_{50}) of all of the synthesized compounds were determined (as shown in Table 4). In general, most of the compounds exhibited greater potency on suspension cell lines (HL-60, K562) than toward attached cell lines (HeLa, A549). For example, compound **6a** showed excellent anti-proliferative activity with an IC_{50} value of 0.69 μ M for the HL-60 cell line, which was equal to the IC_{50} value for the positive control VP-16 and far exceeded the lead compound MsF.

The structure-activity relationships (SARs) showed that the type and position of the substitutions play an important role in determining the anti-proliferative potency (as shown in Fig. 2). First, substitution at the C-9 position was crucial for the cytotoxicity of MsF derivatives. In general, cytotoxicity of the 9-substituted derivatives followed the order $-Br \approx -H > -Cl \approx -F > -NO_2$. The 9-Br-substituted derivatives (*e.g.* **6a**, **6c**, **6d 6e** and **6f**) showed remarkable growth inhibitory activity against most of the tested

Table 4 $\,$ IC_{50} values ($\mu M)$ of the mansonone F derivatives against cancer cells

	$\mathrm{IC}_{50}{}^{a}\left(\mu\mathrm{M} ight)$			
Compd.	A549	HeLa	K562	HL-60
3a	12.74	3.04	0.85	0.62
3 b	30.71	25.22	15.12	5.82
3c	>50	>50	42.32	27.26
3d	9.55	8.33	6.36	0.84
3e	4.24	22.14	7.33	0.44
3f	6.72	17.83	8.27	0.95
4a	33.13	9.95	4.56	1.75
4b	37.34	30.92	5.21	1.37
4 c	32.33	18.32	10.06	2.48
5a	15.52	4.10	2.65	0.74
5b	37.52	32.24	21.14	2.84
5c	28.74	28.52	43.14	3.32
6a	2.65	1.47	0.97	0.69
6b	34.23	28.76	8.74	11.22
6c	27.31	13.98	6.94	2.28
6d	5.63	7.61	7.18	0.93
6e	4.94	6.23	2.26	0.60
6f	5.53	4.61	1.53	0.58
7a	>50	42.82	45.24	6.12
7b	>50	>50	>50	31.13
7 c	>50	>50	>50	35.43
7d	41.17	38.25	36.02	12.26
7e	38.14	29.71	15.32	4.41
7f	>50	>50	20.06	13.96
13	36.36	2.11	2.21	0.59
14	20.25	2.56	2.31	0.58
16a	13.14	9.27	15.42	2.65
16b	14.15	9.67	11.17	1.87
16c	6.07	3.51	5.21	1.92
16d	5.56	2.26	3.71	1.14
17	5.06	2.91	2.91	0.64
MsF	9.77	38.88	10.93	18.44
VP-16	26.43	11.32	1.64	0.49

 a The IC_{50} represents compound concentration giving 50% survival of each cell line.



Fig. 2 Summary of SARs of MsF derivatives.

cell lines. Second, substitution at the C-2 and C-3 positions with flexible alkyl chain (*e.g.*, series **a** with 2,3-diEt, series **d** with 2,3diPr, series **e** with 2,3-diBu, and series **f** with 2,3-diPent) yielded better cytotoxic activity than substitution with a rigid aromatic ring (*e.g.*, series **b** with 2-Ph, 3-Me and series **c** with 2,3-diPh). Alkyl chain length had no influence on cytotoxicity. Finally, compared with the **a** series without 4,5-position substitutions (*e.g.*, **3a–7a**), 4,5-diF substituted derivatives (*e.g.*, **13** and **14**) could maintain cytotoxic activity. However, reduced cytotoxic activity was observed when the 5-fluorine atom of compound **13** was replaced by other amino groups (*e.g.*, series **16**).

Conclusions

In summary, a novel and convenient synthetic route for MsF derivatives was established. The key step for synthesizing MsF derivatives involved the rapid construction of oxaphenalene structure by a one-step reaction of Ru(II)-catalyzed C-H functionalization.

This concise and practical synthetic procedure allows easy induction of a variety of substituents at the C-2, C-3, C-4, C-5 and C-9 positions, and scaled-up production of promising candidate compounds. Furthermore, the cytotoxic activity of synthesized compounds was evaluated in four human cancer cell lines. The SARs of MsF derivatives were discussed in this study and we found that **6a** has excellent anti-proliferative activities which needs to be further studied.

Experimental

General materials and methods

All reactions were monitored by using TLC purchased from Qingdao Haiyang Chemical Co. Ltd. High resolution mass spectra (HRMS) were recorded on Shimadzu LCMS-IT-TOF in electrospray ionization (ESI) positive or negative modes. Melting points (Mp) were determined using a SRS-OptiMelt automated melting point instrument without correction. ¹H (400 MHz), ¹³C NMR (100 MHz), HMBC, HMQC and NOESY were recorded on a Bruker BioSpin GmbH spectrometer with tetramethylsilane (TMS) as an internal standard. IR spectra were recorded on Bruker Equinox 55 Fourier transform spectrometer. The purification of synthesized compounds were carried out by using flash column chromatography with silica gel (200-300 mesh) purchased from Qingdao Haiyang Chemical Co. Ltd. Their purities were proved to be higher than 95% by using analytical HPLC equipped with Shimadzu LC-20AB system and an Ultimate XB-C18 column (4.6 \times 250 mm, 5 μ m) at a flow rate of 0.25 mL min⁻¹. The detection wavelength was 254 nm, and the mobile phase was methanol-water (80:20-60:40) containing 0.1% TFA. The main text of the article should appear here with headings as appropriate.

Cell culture and cell lines. The human acute promyelocytic leukemia cell HL-60, human chronic myelogenous leukemia cell K-562, human cervical cancer cell HeLa, human lung cancer cell A549 were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and preserved at our lab. The HL-60 and K-562 cells were cultured in a RPMI-1640 medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA), and other cell lines were grown in Dulbecco's Modified Eagle's Medium (D-MEM, Gibco Carlsbad, CA) supplemented with 10% fetal bovine serum. All cells were cultured with 5% CO₂ at 37 °C.

Synthetic procedures

General procedure for synthesis of 2a–2f. A suspension of 4nitronaphthalen-1-ol (945.0 mg, 5.00 mmol), alkyne (5 mmol), $[RuCl_2(p-cymene)]_2$ (48 mg, 1.5% mmol) and $Cu(OAc)_2 \cdot H_2O$ (955 mg, 5 mmol), and dimethylformamide (8.0 mL) in ChemGlass pressure vessel was stirred at 100 °C for 9–16 h. After the starting material was consumed up, the reaction mixture was cooled to room temperature, diluted with dichloromethane, and filtered with a short column chromatography on silica gel. The organic layers were washed with water (3 × 30 mL) and dried over Na₂SO₄. After filtration, the filtrate was collected and the solvent was removed under reduced pressure, the crude product was purified by using column chromatography on silica gel (petroleum ether) to give the target compounds.

2,3-Diethyl-7-nitrobenzo[*de*]**chromene (2a).** Reddish yellow solid, yield 75%. ¹H NMR (400 MHz, CDCl₃) δ 8.59 (d, J = 8.9 Hz, 1H), 8.43 (d, J = 8.9 Hz, 1H), 7.66 (dd, J = 8.8, 7.5 Hz, 1H), 7.11 (d, J = 7.4 Hz, 1H), 6.80 (d, J = 8.9 Hz, 1H), 2.57 (m, 4H), 1.28 (t, J = 7.5 Hz, 3H), 1.20 (t, J = 7.5 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 158.2, 153.2, 136.9, 132.0, 130.9, 128.9, 128.5, 122.8, 119.3, 114.4, 114.2, 106.0, 23.7, 19.5, 12.7, 12.3. HRMS (ESI): calcd for C₁₆H₁₅NO₃ [M + H]⁺: 270.1125; found: 270.1117.

3-Methyl-7-nitro-2-phenylbenzo[*de*]**chromene** (2**b**). Orangered solid, yield 88%. ¹H NMR (400 MHz, CDCl₃) δ 8.68 (d, *J* = 8.9 Hz, 1H), 8.47 (d, *J* = 8.9 Hz, 1H), 7.74 (dd, *J* = 8.9, 7.4 Hz, 1H), 7.61–7.58 (m, 2H), 7.56–7.42 (m, 3H), 7.19 (d, *J* = 7.9 Hz, 1H), 6.89 (d, *J* = 8.9 Hz, 1H), 2.17 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 157.9, 149.7, 137.2, 133.3, 132.0, 132.0, 129.5, 129.3, 128.9, 128.4, 128.2, 122.4, 120.3, 115.6, 110.7, 106.9, 13.7. HRMS (ESI): calcd for C₁₉H₁₃NO₃ [M + H]⁺: 304.0968; found: 304.0958.

7-Nitro-2,3-diphenylbenzo[*de*]chromene (2c). Orange-red solid, yield 85%. ¹H NMR (400 MHz, CDCl₃) δ 8.62 (d, J = 8.9 Hz, 1H), 8.49 (d, J = 8.8 Hz, 1H), 7.55 (dd, J = 8.8, 7.6 Hz, 1H), 7.46–7.37 (m, 3H), 7.32–7.29 (m, 2H), 7.27–7.19 (m, 5H), 6.95 (d, J = 8.8 Hz, 1H), 6.78 (d, J = 7.4 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 158.0, 149.3, 137.5, 134.2, 132.8, 132.3, 131.9, 130.7, 129.3, 129.1, 129.0, 128.9, 128.1, 127.9, 122.7, 120.4, 118.3, 117.9, 106.4. HRMS (ESI): calcd for C₂₄H₁₅NO₃ [M + H]⁺: 366.0947; found: 366.0939.

7-Nitro-2,3-dipropylbenzo[*de*]chromene (2d). Deep-red solid, yield 78%. ¹H NMR (400 MHz, CDCl₃) δ 8.49 (d, *J* = 8.8 Hz, 1H), 8.33 (d, *J* = 8.9 Hz, 1H), 7.56 (dd, *J* = 8.8, 7.6 Hz, 1H), 6.99 (d, *J* = 7.4 Hz, 1H), 6.68 (d, *J* = 8.8 Hz, 1H), 2.48–2.37 (m, 4H), 1.71–1.60 (m, 2H), 1.58–1.46 (m, 2H), 0.97 (td, *J* = 7.4, 5.4 Hz, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 158.1, 152.4, 136.8, 132.0, 131.1, 128.8, 128.4, 122.7, 119.3, 114.6, 113.4, 105.9, 32.3, 28.4, 21.2, 21.0, 14.2, 13.8. HRMS (ESI): calcd for C₁₈H₁₉NO₃ [M + H]⁺: 298.1438; found: 298.1418.

2,3-Dibutyl-7-nitrobenzo[*de*]**chromene** (2e). Deep-red oli, yield 74%. ¹H NMR (400 MHz, CDCl₃) δ 8.58 (d, *J* = 8.8 Hz, 1H), 8.41 (d, *J* = 8.9 Hz, 1H), 7.65 (dd, *J* = 8.8, 7.5 Hz, 1H), 7.07 (d, *J* = 7.4 Hz, 1H), 6.78 (d, *J* = 8.9 Hz, 1H), 2.56–2.47 (m, 4H), 1.68 (m, 2H), 1.54–1.42 (m, 6H), 0.99 (m, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 158.1, 152.5, 136.7, 132.0, 131.1, 128.8, 128.4, 122.6, 119.2, 114.5, 113.4, 105.9, 30.2, 30.1, 29.8, 26.1, 22.9, 22.5, 13.9, 13.8. HRMS (ESI): calcd for C₂₀H₂₃NO₃ [M + H]⁺: 326.1751; found: 326.1728.

7-Nitro-2,3-dipentylbenzo[*de*]chromene (2f). Deep-red oil, yield 78%. ¹H NMR (400 MHz, CDCl₃) δ 8.60 (d, *J* = 8.8 Hz, 1H), 8.44 (d, *J* = 8.9 Hz, 1H), 7.67 (dd, *J* = 8.8, 7.5 Hz, 1H), 7.09 (d, *J* = 7.4 Hz, 1H), 6.80 (d, *J* = 8.9 Hz, 1H), 2.58–2.48 (m, 4H), 1.77–1.67

(m, 2H), 1.60–1.57 (m, 2H), 1.47–1.36 (m, 8H), 0.99–0.92 (m, 6H). 13 C NMR (101 MHz, CDCl₃) δ 158.1, 152.5, 136.7, 132.0, 131.1, 128.8, 128.4, 122.6, 119.2, 114.5, 113.4, 105.9, 32.0, 31.5, 30.4, 27.6, 27.4, 26.4, 22.5, 22.4, 14.0, 13.9. HRMS (ESI): calcd for C₂₂H₂₇NO₃ [M + H]⁺: 354.2064; found: 354.2056.

General procedure for synthesis of 3a-3f. Sodium hyposulfite (1.74 g, 10 mmol, 5 equiv.) was added to a solution of nitro-substituted products (2a-2f, 2 mmol) in THF/H₂O (120 mL, 3 : 1). The reaction mixture was heated to 50 °C and further stirred for 2 h at this temperature. After the starting material was consumed up, the reaction mixture was concentrated to remove the THF under reduced pressure and then the residue was extracted with dichloromethane (30 mL \times 3). The combined extracts were evaporated to remove the solvent. The solid residue was dissolved in acetone (60 mL) and a solution of Fremy's salt (1.34 g, 5 mmol, 2.5 equiv.) in 0.06 M KH₂PO₄ solution (68 mL) was added with vigorous stirring. After stirring for 1 h, the mixture was extracted with dichloromethane (30 mL \times 3). The combined extracts were washed with brine (30 mL), dried over Na₂SO₄, filtered and evaporated to dryness. The solid residue was purified by using flash column chromatography (silica gel), eluting with 5% ethyl acetate in dichloromethane to give the target compounds.

2,3-Diethylbenzo[*de*]**chromene-7,8-dione** (3a). Reddish brown solid, yield 65%. Mp: 200.2–203.5 °C; IR ν_{max} (KBr) cm⁻¹: 1639, 1695. ¹H NMR (400 MHz, CDCl₃) δ 8.11 (dd, J = 5.8, 2.8 Hz, 1H), 7.86–7.50 (m, 2H), 6.07 (s, 1H), 2.64 (qd, J = 7.5, 2.4 Hz, 4H), 1.30 (t, J = 7.5 Hz, 3H), 1.22 (t, J = 7.6 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 180.3, 178.1, 166.3, 155.1, 133.2, 131.7, 130.0, 129.9, 129.0, 121.5, 113.1, 104.1, 23.9, 19.3, 14.4, 12.4. HRMS (ESI): calcd for C₁₆H₁₄O₃ [M + Na]⁺: 277.0835; found: 277.0829. HPLC purity: 99.9%.

3-Methyl-2-phenylbenzo[*de*]**chromene-7,8-dione (3b).** Reddish brown solid, yield 71%. Mp: 215.6–218.3 °C; IR ν_{max} (KBr) cm⁻¹: 1633, 1696. ¹H NMR (400 MHz, CDCl₃) δ 8.17 (dd, J = 7.3, 1.3 Hz, 1H), 7.80 (dd, J = 7.3, 1.2 Hz, 1H), 7.75 (t, J = 7.2 Hz, 1H), 7.56–7.50 (m, 5H), 6.11 (s, 1H), 2.30 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 180.1, 178.2, 166.0, 151.1, 133.3, 132.8, 132.3, 130.6, 130.0, 129.8, 129.6, 129.3, 128.5, 121.7, 109.1, 104.5, 13.5. HRMS (ESI): calcd for C₁₉H₁₂O₃ [M + H]⁺: 289.0859; found: 289.0864. HPLC purity: 99.3%.

2,3-Diphenylbenzo[*de*]**chromene-7,8-dione** (3c). Reddish brown solid, yield 55%. Mp: 294.1–296.3 °C; IR ν_{max} (KBr) cm⁻¹: 1627, 1689. ¹H NMR (400 MHz, CDCl₃) δ 8.15 (dd, *J* = 7.5, 1.0 Hz, 1H), 7.58 (t, *J* = 7.8 Hz, 1H), 7.45–7.40 (m, 3H), 7.36 (dd, *J* = 8.1, 1.0 Hz, 1H), 7.32–7.21 (m, 7H), 6.24 (s, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 180.0, 178.3, 165.8, 150.9, 133.1, 132.7, 132.0, 131.7, 131.0, 130.9, 129.6, 129.5, 129.2, 129.0, 128.5, 128.1, 121.3, 116.5, 104.7. HRMS (ESI): calcd for C₂₄H₁₄O₃ [M + H]⁺: 351.1016; found: 351.1016. HPLC purity: 99.7%.

2,3-Dipropylbenzo[*de*]chromene-7,8-dione (3d). Reddish brown solid, yield 65%. Mp: 165.6–168.4 °C; IR ν_{max} (KBr) cm⁻¹: 1631, 1696. ¹H NMR (400 MHz, CDCl₃) δ 8.11 (dd, *J* = 4.6, 4.0 Hz, 1H), 7.70 (m, 2H), 6.06 (s, 1H), 2.64–2.54 (m, 4H), 1.75 (m, 2H), 1.61 (m, 2H), 1.07 (m, 6H). ¹³C NMR (400 MHz, CDCl₃) δ 180.3, 178.1, 166.3, 154.3, 133.1, 132.0, 130.0, 129.94, 129.2, 121.5, 112.3, 104.1, 32.4, 28.1, 23.1, 21.1, 14.1, 13.8. HRMS (ESI): calcd

for $C_{18}H_{18}O_3 [M + H]^+$: 283.1329; found: 283.1325. HPLC purity: 97.9%.

2,3-Dibutylbenzo[*de*]**chromene-7,8-dione (3e).** Reddish brown solid, yield 60%. Mp: 160.7–162.6 °C; IR ν_{max} (KBr) cm⁻¹: 1629, 1699. ¹H NMR (400 MHz, CDCl₃) δ 8.10 (dd, J = 5.2, 3.4 Hz, 1H), 7.77–7.46 (m, 2H), 6.05 (s, 1H), 2.67–2.35 (m, 4H), 1.80–1.59 (m, 2H), 1.59–1.27 (m, 6H), 0.99 (m, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 180.4, 178.1, 166.3, 154.4, 133.1, 132.0, 130.0, 129.9, 129.1, 121.5, 112.2, 104.1, 32.0, 30.3, 29.9, 25.8, 22.7, 22.4, 13.8, 13.8. HRMS (ESI): calcd for C₂₀H₂₂O₃ [M + H]⁺: 311.1642; found: 311.1637. HPLC purity: 95.3%.

2,3-Dipentylbenzo[*de*]**chromene-7,8-dione (3f).** Reddish brown solid, yield 66%. Mp: 153.6–155.1 °C; IR ν_{max} (KBr) cm⁻¹: 1627, 1699.¹H NMR (400 MHz, CDCl₃) δ 8.07 (dd, J = 5.3, 3.3 Hz, 1H), 7.73–7.52 (m, 2H), 6.03 (s, 1H), 2.61–2.43 (m, 4H), 1.73–1.65 (m, 2H), 1.61–1.48 (m, 2H), 1.47–1.32 (m, 8H), 0.95–0.92 (m, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 180.3, 178.1, 166.3, 154.4, 133.1, 132.0, 130.0, 129.9, 129.2, 121.4, 112.3, 104.1, 31.8, 31.4, 30.5, 29.6, 27.5, 26.1, 22.4, 22.3, 13.9, 13.9. HRMS (ESI): calcd for C₂₂H₂₆O₃ [M + H]⁺: 339.1955; found: 339.1954. HPLC purity: 97.1%.

General procedure for synthesis of 4a–4c. Selectfluor (1 mmol, 2 equiv.) was added to a solution of *o*-quinone products (3a–3c, 0.5 mmol) dissolved in anhydrous acetonitrile. The reaction mixture was heated at 80 $^{\circ}$ C with stirring for 4 h. After the starting material was consumed up, the reaction mixture was filtered and the filtrate was concentrated under reduced pressure. The solid residue was purified by using flash column chromatography (silica gel), eluting with dichloromethane (DCM) to give the target compounds.

2,3-Diethyl-9-fluorobenzo[*de*]**chromene-7,8-dione** (4a). Reddish brown solid, yield 19%. Mp: 212.4–213.8 °C; IR ν_{max} (KBr) cm⁻¹: 1634, 1695. ¹H NMR (400 MHz, CDCl₃) δ 8.06 (dd, J = 7.2, 1.3 Hz, 1H), 7.70–7.59 (m, 2H), 2.68 (q, J = 7.6, 2H), 2.64 (q, J = 7.6, 2H), 1.32 (t, J = 7.5 Hz, 3H), 1.22 (t, J = 7.6 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 179.1 (d, J = 6.5 Hz), 169.4 (d, J = 9.0 Hz), 154.9, 151.8 (d, J = 8.4 Hz), 142.1 (d, J = 253.4 Hz), 132.8, 131.6 (d, J = 6.7 Hz), 130.7, 129.7, 127.8, 119.3, 113.6, 23.9, 19.3, 14.4, 12.2. HRMS (ESI): calcd for C₁₆H₁₃O₃F [M + Na]⁺: 295.0741; found: 295.0743. HPLC purity: 95.4%.

9-Fluoro-3-methyl-2-phenylbenzo[*de*]chromene-7,8-dione (4b). Reddish brown solid, yield 16%. Mp: 216.7–219.3 °C; IR ν_{max} (KBr) cm⁻¹: 1632, 1699. ¹H NMR (400 MHz, CDCl₃) δ 8.11 (d, *J* = 7.4 Hz, 1H), 7.78 (d, *J* = 8.0 Hz, 1H), 7.68 (t, *J* = 7.8 Hz, 1H), 7.63–7.56 (m, 2H), 7.54–7.47 (m, 3H), 2.31 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 178.1 (d, *J* = 6.8 Hz), 169.5 (d, *J* = 9.4 Hz), 150.2 (d, *J* = 8.2 Hz), 149.2, 140.0 (d, *J* = 249.4 Hz), 133.0, 131.8, 131.6 (d, *J* = 6.4 Hz), 130.4, 130.2, 130.0, 129.2, 128.6, 127.7, 118.9, 109.6, 13.2. HRMS (ESI): calcd for C₁₉H₁₁O₃F [M + H]⁺: 307.0765; found: 307.0763. HPLC purity: 96.2%.

9-Fluoro-2,3-diphenylbenzo[*de*]chromene-7,8-dione (4c). Reddish brown solid, yield 20%. Mp: 242.3–245.7 °C; IR ν_{max} (KBr) cm⁻¹: 1630, 1708. ¹H NMR (400 MHz, CDCl₃) δ 8.12 (dd, J = 7.5, 1.1 Hz, 1H), 7.54 (t, J = 7.8 Hz, 1H), 7.46 (dd, J = 6.5, 3.9 Hz, 3H), 7.38–7.30 (m, 6H), 7.26 (d, J = 2.0 Hz, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 178.9 (d, J = 6.3 Hz), 170.0 (d, J = 9.7 Hz), 151.2 (d, J = 8.3 Hz), 150.4, 140.8 (d, J = 255.6 Hz), 132.8, 132.6, 132.3, 131.5, 131.0, 129.8, 129.4 (d, J = 7.9 Hz), 129.3, 129.0, 128.7, 128.3, 128.2, 127.4, 119.1, 116.7. HRMS

(ESI): calcd for $C_{24}H_{13}O_3F [M + H]^+$: 369.0921; found: 369.0929. HPLC purity: 96.0%.

General procedure for synthesis of 5a–5c. *N*-Chlorosuccinimide (0.6 mmol, 1.2 equiv.) and benzoyl peroxide (12 mg) were added to a solution of *o*-quinone products (**3a–3c**, 0.5 mmol) in carbon tetrachloride. The reaction mixture was heated at 75 °C with stirring. After the starting material was consumed up, the reaction mixture was filtered and the filtrate was concentrated under reduced pressure. The solid residue was purified by using flash column chromatography (silica gel), eluting with dichloromethane to give the target compounds.

9-Chloro-2,3-diethylbenzo[*de*]**chromene-7,8-dione** (5a). Reddish brown solid, yield 55%. Mp: 209.3–211.4 °C; IR ν_{max} (KBr) cm⁻¹: 1634, 1695. ¹H NMR (400 MHz, CDCl₃) δ 8.12 (dd, J = 7.1, 1.3 Hz, 1H), 7.82–7.64 (m, 2H), 2.74 (q, J = 7.5 Hz, 2H), 2.67 (q, J = 7.5 Hz, 2H), 1.36 (t, J = 7.5 Hz, 3H), 1.22 (t, J = 7.6 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 178.3, 172.4, 160.8, 155.4, 133.2, 131.5, 130.8, 130.7, 129.5, 128.6, 121.0, 113.9, 23.9, 19.3, 14.3, 11.9. HRMS (ESI): calcd for C₁₆H₁₃O₃Cl [M + H]⁺: 289.0626; found: 289.0627. HPLC purity: 98.8%.

9-Chloro-3-methyl-2-phenylbenzo[*de*]**chromene-7,8-dione (5b).** Reddish brown solid, yield 47%. Mp: 223.4–225.6 °C; IR ν_{max} (KBr) cm⁻¹: 1638, 1693. ¹H NMR (400 MHz, CDCl₃) δ 8.18 (dd, *J* = 7.4, 1.1 Hz, 1H), 7.84 (dd, *J* = 8.1, 1.1 Hz, 1H), 7.75 (t, *J* = 7.6 Hz, 1H), 7.68–7.64 (m, 2H), 7.56–7.51 (m, 3H), 2.38 (s, 3H). ¹³C NMR (101 MHz, Pyr) δ 178.2, 172.9, 160.1, 150.5, 133.1, 132.5, 132.4, 130.3, 130.1, 129.5, 129.2, 128.7, 121.3, 110.0, 65.5, 13.1. HRMS (ESI): calcd for C₁₉H₁₁O₃Cl [M + H]⁺: 323.0469; found: 323.0461. HPLC purity: 98.9%.

9-Chloro-2,3-diphenylbenzo[*de*]chromene-7,8-dione (5c). Reddish brown solid, yield 52%. Mp: 243.6–245.1 °C; IR ν_{max} (KBr) cm⁻¹: 1638, 1692. ¹H NMR (400 MHz, CDCl₃) δ 8.17 (dd, *J* = 7.4, 0.8 Hz, 1H), 7.58 (t, *J* = 7.8 Hz, 1H), 7.50–7.46 (m, 3H), 7.42–7.36 (m, 3H), 7.32 (d, *J* = 7.2 Hz, 1H), 7.28–7.24 (m, 4H), 7.24 (d, *J* = 1.6 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 178.2, 172.7, 160.4, 150.6, 133.1, 132.8, 132.7, 132.3, 131.5, 131.4, 130.9, 129.9, 129.4, 128.9, 128.8, 128.2, 120.7, 117.0. HRMS (ESI): calcd for C₂₄H₁₃O₃Cl [M + H]⁺: 385.0626; found: 385.0637. HPLC purity: 99.7%.

General procedure for the preparation of 6a–6f. *N*-Bromosuccinimide (0.6 mmol, 1.2 equiv.) and benzoyl peroxide (12 mg) were added to a solution of *o*-quinone products (**3a–3f**, 0.5 mmol) dissolved in carbon tetrachloride. The reaction mixture was heated at 75 °C with stirring. After the starting material was consumed up, the reaction mixture was filtered and concentrated under reduced pressure. The solid residue was purified by using flash column chromatography (silica gel), eluting with dichloromethane to give the target compounds.

9-Bromo-2,3-diethylbenzo[*de*]**chromene-7,8-dione (6a).** Reddish brown solid, yield 48%. Mp: 242.8–244.7 °C; IR ν_{max} (KBr) cm⁻¹: 1632, 1695. ¹H NMR (400 MHz, CDCl₃) δ 8.13 (dd, *J* = 6.5, 2.0 Hz, 1H), 7.78–7.62 (m, 2H), 2.74 (q, *J* = 7.5 Hz, 2H), 2.67 (q, *J* = 7.6 Hz, 2H), 1.37 (t, *J* = 7.5 Hz, 3H), 1.22 (t, *J* = 7.6 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 177.9, 172.6, 162.3, 155.5, 133.4, 131.5, 130.7, 129.6, 128.6, 121.2, 114.1, 99.6, 23.9, 19.3, 14.3, 11.9. HRMS (ESI): calcd for C₁₆H₁₃O₃Br [M + H]⁺: 333.0121; found: 333.0124. HPLC purity: 99.2%.

9-Bromo-3-methyl-2-phenylbenzo[*de*]chromene-7,8-dione (6b). Reddish brown solid, yield 50%. Mp: 245.4–247.3 °C; IR ν_{max} (KBr) cm⁻¹: 1633, 1690. ¹H NMR (400 MHz, CDCl₃) δ 8.19 (dd, J = 7.4, 1.1 Hz, 1H), 7.84 (dd, J = 8.1, 1.1 Hz, 1H), 7.77 (t, J = 7.6 Hz, 1H), 7.71–7.66 (m, 2H), 7.57–7.51 (m, 3H), 2.39 (s, 3H). ¹³C NMR (101 MHz, Pyr) δ 177.6, 173.2, 161.6, 150.3, 133.2, 132.4, 130.2, 130.1, 129.5, 129.2, 128.7, 123.7, 122.7, 121.3, 110.1, 100.0, 13.2. HRMS (ESI): calcd for C₁₉H₁₁O₃Br [M + H]⁺: 366.9964; found: 366.9961. HPLC purity: 98.0%.

9-Bromo-2,3-diphenylbenzo[*de*]chromene-7,8-dione (6c). Reddish brown solid, yield 45%. Mp: 271.4–273.3 °C; IR ν_{max} (KBr) cm⁻¹: 1641, 1699. ¹H NMR (400 MHz, CDCl₃) δ 8.17 (dd, J = 7.5, 1.1 Hz, 1H), 7.60 (m, J = 7.6 Hz, 1H), 7.48 (m, 3H), 7.44–7.40 (m, 2H), 7.38 (dd, J = 8.2, 1.1 Hz, 1H), 7.33–7.27 (m, 5H). ¹³C NMR (101 MHz, CDCl₃) δ 178.2, 177.7, 172.9, 162.0, 150.8, 133.2, 132.8, 132.82, 132.3, 131.5, 130.9, 129.9, 129.5, 129.0, 128.9, 128.2, 121.0, 117.1, 100.1, 100.0. HRMS (ESI): calcd for C₂₄H₁₃O₃Br [M + H]⁺: 429.0121; found: 429.0120. HPLC purity: 96.8%.

9-Bromo-2,3-dipropylbenzo[*de*]**chromene-7,8-dione** (6d). Reddish brown solid, yield 46%. Mp: 256.4–257.7 °C; IR ν_{max} (KBr) cm⁻¹: 1633, 1693. ¹H NMR (400 MHz, CDCl₃) δ 8.11 (dd, *J* = 5.3, 3.2 Hz, 1H), 7.78–7.65 (m, 2H), 2.69 (t, *J* = 7.2 Hz, 2H), 2.61 (t, *J* = 7.2 Hz, 2H), 1.93–1.75 (m, 2H), 1.60 (m, 2H), 1.07 (t, *J* = 7.4 Hz, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 177.9, 172.6, 162.3, 154.8, 133.3, 131.8, 130.7, 129.7, 128.7, 121.2, 113.2, 99.6, 32.4, 28.0, 23.0, 20.7, 14.0, 13.8. HRMS (ESI): calcd for C₁₈H₁₇O₃Br [M + H]⁺: 361.0434; found: 361.0432. HPLC purity: 99.2%.

9-Bromo-2,3-dibutylbenzo[*de*]**chromene-7,8-dione (6e).** Reddish brown solid, yield 80%. Mp: 274.3–275.7 °C; IR ν_{max} (KBr) cm⁻¹: 1635, 1696. ¹H NMR (400 MHz, CDCl₃) δ 8.04 (t, J = 4.8, 1H), 7.69–7.56 (m, 2H), 2.63 (t, J = 7.2, 2H), 2.55 (t, J = 7.2, 2H), 1.74–1.66 (m, 2H), 1.55–1.31 (m, 6H), 0.94–0.90 (m, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 177.9, 172.6, 162.3, 154.9, 133.3, 131.8, 130.7, 129.8, 128.6, 121.0, 113.3, 99.5, 31.9, 30.2, 29.4, 25.8, 22.7, 22.4, 13.8, 13.8. HRMS (ESI): calcd for C₂₀H₂₁O₃Br [M + H]⁺: 389.0747; found: 389.0738. HPLC purity: 98.8%.

9-Bromo-2,3-dibutylbenzo[*de*]**chromene-7,8-dione (6f).** Reddish brown solid, yield 52%. Mp: 273.4–275.7 °C; IR ν_{max} (KBr) cm⁻¹: 1632, 1689. ¹H NMR (400 MHz, CDCl₃) δ 8.05 (t, *J* = 4.3 Hz, 1H), 7.64 (d, *J* = 4.5 Hz, 2H), 2.63 (t, *J* = 7.2 Hz, 2H), 2.54 (t, *J* = 7.2 Hz, 2H), 1.78–1.68 (m, 2H), 1.50–1.48 (m, 4H), 1.36–1.28 (m, 6H), 0.88–0.84 (m, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 178.0, 172.7, 162.3, 154.9, 133.3, 131.8, 130.7, 129.7, 128.7, 121.2, 113.3, 99.6, 31.8, 31.3, 30.4, 29.6, 26.9, 26.1, 22.4, 22.3, 13.9, 13.8. HRMS (ESI): calcd for C₂₂H₂₅O₃Br [M + H]⁺: 417.1060; found: 417.1065. HPLC purity: 96.9%.

General procedure for synthesis of 7a–7f. *O*-Quinone product (3a–3f, 0.5 mmol) was dissolved in acetic anhydride (25 mL) and cooled to 0 °C in an ice bath. Cupric nitrate (0.6 mmol, 1.2 equiv.) was added under stirring. The reaction mixture was warmed up to room temperature, and stirring was continued until the reaction was completed. The mixture was slowly poured into chopped ice, and the resulting mixture was extracted with DCM. The combined organic layer was washed with water (3 times) and concentrated to give a crude product under reduced pressure. The crude product was purified by

using column chromatography (silica gel), eluting with dichloromethane to give the target compound.

2,3-Diethyl-9-nitrobenzo[*de*]**chromene-7,8-dione** (7a). Deep brown solid, yield 65%. Mp: 198.4–200.1 °C; IR ν_{max} (KBr) cm⁻¹: 1643, 1698. ¹H NMR (400 MHz, DMSO) δ 8.20 (d, J = 7.7 Hz, 2H), 8.09 (t, J = 7.2 Hz, 1H), 2.89–2.80 (m, 4H), 1.28 (t, J = 7.5 Hz, 3H), 1.22 (t, J = 7.4 Hz, 3H). ¹³C NMR (101 MHz, DMSO) δ 176.7, 167.9, 158.9, 155.1, 135.6, 132.7, 130.2, 130.0, 129.5, 129.4, 116.8, 116.0, 23.0, 18.5, 14.3, 11.9. HRMS (ESI): calcd for C₁₆H₁₃NO₅ [M + H]⁺: 300.0866; found: 300.0872. HPLC purity: 99.4%.

3-Methyl-9-nitro-2-phenylbenzo[*de*]chromene-7,8-dione (7b). Deep brown solid, yield 64%. Mp: 275.4–276.3 °C; IR ν_{max} (KBr) cm⁻¹: 1658, 1702. ¹H NMR (400 MHz, DMSO) δ 8.23 (d, J = 8.0 Hz, 1H), 8.22 (d, J = 7.6 Hz, 1H), 8.10 (t, J = 7.8 Hz, 1H), 7.69 (dd, J = 7.8, 1.8 Hz, 2H), 7.63–7.59 (m, 3H), 2.43 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 177.5, 168.6, 159.3, 150.8, 136.1, 134.5, 131.7, 131.2, 131.1, 130.8, 130.3, 129.8, 129.6, 129.2, 117.7, 112.8, 13.7. HRMS (ESI): calcd for C₁₉H₁₁NO₅ [M + H]⁺: 334.0710; found: 334.0736. HPLC purity: 95.2%.

9-Nitro-2,3-diphenylbenzo[*de*]chromene-7,8-dione (7c). Deep brown solid, yield 71%. Mp: 284.5–286.7 °C; IR ν_{max} (KBr) cm⁻¹: 1657, 1696. ¹H NMR (400 MHz, CDCl₃) δ 8.33 (dd, J = 7.5, 1.0 Hz, 1H), 7.82 (t, J = 7.6 Hz, 1H), 7.59 (dd, J = 8.3, 1.0 Hz, 1H), 7.55– 7.49 (m, 3H), 7.41–7.28 (m, 7H). ¹³C NMR (101 MHz, CDCl₃) δ 181.7, 173.6, 163.7, 155.2, 140.9, 139.0, 137.3, 136.35, 136.2, 136.0, 135.3, 135.2, 134.8, 134.5, 134.2, 134.1, 133.6, 123.6, 122.1. HRMS (ESI): calcd for C₂₄H₁₃NO₅ [M + H]⁺: 396.0866; found: 396.0885. HPLC purity: 95.4%.

9-Nitro-2,3-dipropylbenzo[*de*]chromene-7,8-dione (7d). Deep brown solid, yield 67%. Mp: 196.5–197.4 °C; IR ν_{max} (KBr) cm⁻¹: 1647, 1699. ¹H NMR (400 MHz, DMSO) δ 8.14 (d, J = 7.3 Hz, 2H), 8.02 (t, J = 7.2 Hz, 1H), 2.79–2.70 (m, 4H), 1.79–1.62 (m, 2H), 1.55 (m, 2H), 0.96 (m, 6H). ¹³C NMR (101 MHz, DMSO) δ 176.8, 167.9, 158.9, 154.3, 135.5, 132.9, 130.2, 130.0, 129.8, 129.4, 116.8, 115.1, 31.3, 27.0, 22.7, 20.4, 13.6, 13.2. HRMS (ESI): calcd for C₁₈H₁₇NO₅ [M + H]⁺: 328.1179; found: 328.1182. HPLC purity: 99.3%.

2,3-Dibutyl-9-nitrobenzo[*de*]chromene-7,8-dione (7e). Deep brown solid, yield 70%. Mp: 159.8–161.4 °C; IR ν_{max} (KBr) cm⁻¹: 1650, 1705. ¹H NMR (400 MHz, DMSO) δ 8.13 (d, J = 7.6 Hz, 1H), 8.11 (d, J = 8.8 Hz, 1H), 8.03 (t, J = 7.6 Hz, 1H), 2.78–2.73 (m, 4H), 1.71–1.56 (m, 2H), 1.50–1.40 (m, 4H), 1.38–1.34 (m, 2H), 0.95–0.91 (m, 6H). ¹³C NMR (101 MHz, DMSO) δ 176.7, 167.9, 158.8, 154.4, 135.6, 132.9, 130.1, 129.7, 129.4, 128.5, 116.7, 115.2, 31.6, 29.2, 29.0, 24.9, 21.9, 21.4, 13.7, 13.6. HRMS (ESI): calcd for C₂₀H₂₁NO₅ [M + H]⁺: 356.1492; found: 356.1491. HPLC purity: 99.6%.

9-Bromo-2,3-dibutylbenzo[*de*]**chromene-7,8-dione (7f).** Deep brown solid, yield 68%. Mp: 149.7–152.8 °C; IR ν_{max} (KBr) cm⁻¹: 1644, 1692. ¹H NMR (400 MHz, DMSO) δ 8.16–8.09 (m, 2H), 8.03 (t, *J* = 7.6 Hz, 1H), 2.75 (t, *J* = 7.3 Hz, 4H), 1.72–1.61 (m, 2H), 1.57–1.46 (m, 2H), 1.43–1.30 (m, 8H), 0.88 (t, *J* = 7.1 Hz, 6H). ¹³C NMR (101 MHz, DMSO) δ 176.7, 167.9, 158.8, 154.4, 135.6, 132.9, 130.0, 129.7, 129.4, 128.5, 116.7, 115.2, 30.9, 30.4, 29.4, 29.1, 26.5, 25.1, 21.9, 21.7, 13.8, 13.6. HRMS (ESI): calcd for $C_{22}H_{25}NO_5 [M + H]^+$: 384.1805; found: 384.1791. HPLC purity: 99.2%.

6,7-Difluoro-1,4-dihydro-1,4-epoxynaphthalene (9). A 500 mL, 3-neck, round-bottom flask, which was equipped with a condenser, rubber seal, gas adapter under N2, was charged 50 mL of THF, 150 mmol furan and 1.44 g of Mg (60 mmol, 1.2 equiv.). The solution which was heated to 75 °C, was added 8 (10.55 g, 50 mmol) in 30 mL of THF via gastight syringe over a 1 h period at such a rate to keep the reaction mixture. The reaction mixture was then stirred for 8 h at 75 °C. Then 20 mL of water was poured into the reaction mixture to halt the reaction, and the aqueous phase was washed $(3 \times 50 \text{ mL})$ with ether. The combined organic phases were dried over Na₂SO₄, vacuum filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (petroleum ether/EtOAc = 20 : 1, 10 : 1, 8 : 1, 5 : 1) to afford 9 (4.89 g, 31%) as yellow oily liquid. ¹H NMR (400 MHz, CDCl₃) δ 7.07 (t, J =7.7 Hz, 2H), 7.03 (t, J = 1 Hz, 2H), 5.69 (s, 2H). ¹³C NMR (101 MHz, $CDCl_3$) δ 148.5 (d, J = 14.9), 146.0 (d, J = 14.9), 145.2 (t, J =4.6 HZ), 143.1, 110.9 (m), 82.1.

6,7-Difluoronaphthalen-1-ol (10). To a stirred solution of **9** (5.76 g, 1.5 mmol) in CH₂Cl₂ (30 mL), diluted BF₃·Et₂O (4.84 mL) was added slowly and stirred for 1 h at 0 °C. After the starting material was consumed up, the solution was washed with water (40 mL) and dried over Na₂SO₄, and then the solvent was removed under educed pressure. The residue was purified by silica gel column chromatography (petroleum ether/EtOAc = 10 : 1) to afford **10** (4.89 g, 84.9%) as white solid. ¹H NMR (400 MHz, CDCl₃) δ 7.94 (t, *J* = 9.5 Hz, 1H), 7.52 (t, *J* = 9.0 Hz, 1H), 7.35 (d, *J* = 8.4 Hz, 1H), 7.32–7.27 (m, 2H) 6.80 (d, *J* = 7.1 Hz, 1H), 5.36 (bs, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 150.9 (d, *J* = 5.1 Hz), 150.5 (dd, *J* = 249.6, 14.7 Hz), 149.6 (dd, *J* = 248.5, 15.2 Hz), 131.8 (d, *J* = 7.7 Hz), 126.4 (d, *J* = 2.3 Hz), 121.1 (d, *J* = 6.8 Hz), 119.9 (dd, *J* = 4.7, 1.6 Hz), 113.3 (d, *J* = 16.77 Hz), 108.7 (d, *J* = 5.8 Hz), 106.6 (d, *J* = 10.3 Hz).

2,3-Diethyl-4,5-difluorobenzo[de]chromene (11). A suspension of 10 (900 mg, 5.00 mmol), 3-hexyne (5 mmol), [RuCl₂(pcymene)]2 (48 mg, 1.5% mmol) and Cu(OAc)2 · H2O (955 mg, 5 mmol), and m-xylene (8.0 mL) in ChemGlass pressure vessel was stirred at 80-100 °C overnight. After the starting material was consumed up, the reaction mixture was cooled to room temperature, diluted with dichloromethane, and filtered with a short column chromatography on silica gel. The organic layers were washed with water (3 \times 30 mL) and dried over Na₂SO₄. After filtration, the filtrate was collected and the solvent was removed under reduced pressure, the crude product was purified by using column chromatography on silica gel (petroleum ether) to give **11** (54%) as pale yellow oily liquid. ¹H NMR (400 MHz, CDCl_3) δ 7.24 (t, J = 8.0 Hz, 1H), 7.10 (dd, J = 8.1, 0.6 Hz, 1H), 7.03 (dd, *J* = 11.1, 7.2 Hz, 1H), 6.71 (d, *J* = 7.7 Hz, 1H), 2.59 (qd, J = 7.4, 2.2 Hz, 2H), 2.46 (q, J = 7.5 Hz, 2H), 1.26 (t, J =7.2 Hz, 3H), 1.20 (td, J = 7.4, 1.0 Hz, 3H). ¹³C NMR (101 MHz, $CDCl_3$) δ 154.7 (d, J = 2.2 Hz), 152.3 (dd, J = 248.2, 17.4 Hz), 152.1 (dd, *J* = 4.7, 1.9 Hz), 140.8 (dd, *J* = 247.3, 17.5 Hz), 131.57 (d, J = 5.5 Hz), 129.0, 127.4 (d, J = 1.6 Hz), 126.3, 120.2 (d, J = 5.1 Hz)Hz), 118.4 (dd, *J* = 5.0, 1.7 Hz), 110.0 (dd, *J* = 5.4, 3.2 Hz), 107.8 (d, *J* = 18.2 Hz), 106.7 (d, *J* = 2.1 Hz), 23.5, 21.4 (d, *J* = 9.6 Hz), 14.5 (d, *J* = 4.2 Hz), 12.4.

2,3-Diethyl-4,5-difluoro-7-nitrobenzo[de]chromene (12). 5 mmol (1.3 g) of 11 was dissolved in acetic anhydride (40 mL) and cooled to 0 °C in an ice bath. 1.21 g of cupric nitrate (5 mmol, 1 equiv.) was added slowly with vigorous stirring. The reaction was warmed to room temperature, and stirring was continued until the reaction was completed. The mixture was diluted with brash ice (300 g), stirred for another 2 h, and filtered to collect the residue. The residue was subjected to chromatography column (silica gel), eluting with 30% dichloromethane in petroleum to give 12 (4.7 g, 73.09%) as yellow solid. ¹H NMR (400 MHz, CDCl₃) δ 8.47 (m, 1H), 8.43 (d, J = 8.9 Hz, 1H), 6.79 (d, J = 8.9 Hz, 1H), 2.69–2.65 (m, 2H), 2.56 (q, J = 7.6 Hz, 2H), 1.28 (t, J = 7.6 Hz, 3H), 1.22 (t, J = 7.4 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 157.2 (d, J = 2.7 Hz), 155.0 (d, J =2.2 Hz), 154.6 (dd, J = 251.8, 16.2 Hz), 141.4 (dd, J = 251.9, 16.9 Hz), 136.5 (d, J = 5.5 Hz), 129.2, 125.8 (dd, J = 11.1, 1.8 Hz), 123.6 (d, J = 2.2 Hz), 120.4 (d, J = 4.7 Hz), 119.6 (dd, J = 7.3, 2.4 Hz), 11.6 (dd, J = 5.7, 3.5 Hz), 109.2 (d, J = 18.9 Hz), 106.8 (d, J = 24.0 Hz), 106.5, 23.5, 21.4 (d, *J* = 10.4 Hz), 14.5 (d, *J* = 4.5 Hz). HRMS (ESI): calcd for $C_{16}H_{13}NO_3F_2$, $[M + H]^+$: 306.0936; found: 306.0940.

2,3-Diethyl-4,5-difluorobenzo[de]chromene-7,8-dione (13). Sodium hyposulfite (1.74 g, 10 mmol, 5 equiv.) was added to a solution of 12 (2 mmol) in THF/H₂O (120 mL, 3:1). The reaction mixture was heated to 50 $^\circ$ C, and further stirred for 2 h at this temperature. After the starting material was consumed up, the reaction mixture was concentrated to remove the THF under reduced pressure and then the residue was extracted with dichloromethane (30 mL \times 3). The combined extracts were evaporated to remove the solvent. The solid residue was then dissolved in acetone (60 mL) and a solution of Fremy's salt (1.34 g, 5 mmol, 2.5 equiv.) in 0.06 M KH₂PO₄ solution (68 mL) was added with vigorous stirring. After stirring for 1 h, the mixture was extracted with dichloromethane (30 mL \times 3). The combined extracts were washed with brine (30 mL), dried over Na₂SO₄, filtered and evaporated to dryness. The solid residue was purified by using flash column chromatography (silica gel), eluting with 5% ethyl acetate in dichloromethane to give 13 (45.19%) as purple black solid. Mp: 145.2–148.5 °C. IR ν_{max} (KBr) cm⁻¹: 1641, 1699. ¹H NMR (400 MHz, CDCl₃) δ 7.92 (t, J = 7.6 Hz, 1H), 6.06 (s, 1H), 2.71 (dq, J = 7.2, 2.4 Hz, 2H), 2.64 (q, J = 7.5 Hz, 2H), 1.29 (t, J = 7.5 Hz, 3H), 1.22 (t, J = 7.4 Hz, 3H). ¹³C NMR (101 MHz, CDCl3) δ 178.1, 177.6, 164.3, 156.6, 153.8 (dd, J = 258.9, 148 Hz), 150.5 (dd, J = 267.0, 14.7 Hz), 127.1 (d, J = 5.1 Hz), 123.2 (d, J = 8.9 Hz), 119.8 (d, J = 5.2 Hz), 119.6 (d, J = 20.3 Hz), 110.7 (d, J = 5.2 Hz), 104.3, 23.8, 21.1 (d, J = 10.9 Hz), 14.9 $(d, J = 4.0 \text{ Hz}), 12.5. \text{ HRMS (ESI): calcd for } C_{16}H_{12}O_3F_2 [M + H]^+$: 291.0827; found: 291.0832. HPLC purity: 99.9%.

9-Bromo-2,3-diethyl-4,5-difluorobenzo[*de*]chromene-7,8-dione (14). *N*-Bromosuccinimide (0.6 mmol, 1.2 equiv.) and benzoyl peroxide (12 mg) were added to a solution of 13 (0.5 mmol) dissolved in carbon tetrachloride. The reaction mixture was heated at 75 $^{\circ}$ C with stirring. After the starting material was consumed up, the reaction mixture was filtered and concentrated under reduced pressure. The solid residue was purified

by using flash column chromatography (silica gel), eluting with dichloromethane to give 14 (74%) as purple black solid. Mp: 304.9–307.4 °C. IR ν_{max} (KBr) cm⁻¹: 1646, 1697. ¹H NMR (400 MHz, $CDCl_3$) δ 7.94 (t, J = 8.0 Hz, 1H), 2.72–2.78 (m, 4H), 1.37 (t, J = 7.4 Hz, 3H), 1.23 (t, J = 7.6 Hz, 3H). ¹³C NMR (101 MHz, $CDCl_3$) δ 175.8, 172.2, 160.3, 156.8, 153.7 (d, J = 259.1, 15.7 Hz), 150.8 (dd, J = 268.2, 14.7 Hz), 125.8 (m)1, 123.1 (d, J = 9.9 Hz), 120.2 (d, I = 20.0 Hz), 119.6 (m), 11.7 (m), 100.1, 23.8, 21.2 (d, J = 11.1 Hz), 14.8 (d, J = 3.4 Hz), 11.9. HRMS (ESI): calcd for $C_{16}H_{11}O_{3}F_{2}Br [M + H]^{+}$: 368.9932; found: 368.9944. HPLC purity: 99.1%.

General procedure for synthesis of compounds 16. A solution of K₂CO₃ (552 mg, 4 mmol), 12 (2 mmol), different amine (6 mmol) in DMF (20 mL) was stirred in 60 °C for 12 h. Then H₂O (40 mL) was added and extracted with CH_2Cl_2 (3 \times 30 mL). The combined organic phases were dried over Na₂SO₄, and the solvent was removed under reduced pressure to get the crude product. The next step was similar to the synthesis of 13.

2,3-Diethyl-4-fluoro-5-(piperidin-1-yl)benzo[de]chromene-7,8dione (16a). 16a was prepared from K₂CO₃ (552 mg, 4 mmol), 12 (2 mmol), piperidin (6 mmol), purple black solid, yield 65%. Mp: 167.9–169.4 °C. IR $\nu_{\rm max}$ (KBr) cm $^{-1}\!\!:$ 1630, 1696. $^1\!{\rm H}$ NMR (400 MHz, $CDCl_3$) δ 7.76 (t, J = 5.4 Hz, 1H), 5.94 (d, J = 6.1 Hz, 1H), 3.20 (d, J = 4.2 Hz, 4H), 2.70 (s, 2H), 2.64–2.58 (m, 2H), 1.75 (s, 4H), 1.65 (s, 2H), 1.29–1.24 (m, 3H), 1.20 (d, J = 6.8 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 179.9, 177.9, 165.7, 155.5, 153.4 (d, *J* = 262.9 Hz), 145.4 (d, J = 10.1 Hz), 126.6 (d, J = 3.9 Hz), 122.6 (d, J = 6.0 Hz), 121.3 (d, I = 12 Hz), 115.5 (d, I = 5.7 Hz), 111.4 (d, I = 4.3Hz), 102.4, 51.6 (d, J = 4.4 Hz), 25.9, 24.0, 23.9, 21.6 (d, J = 12.1 Hz), 15.1 (d, J = 4.4 Hz), 12.5. HRMS (ESI): calcd for $C_{20}H_{20}NO_4F$ $[M + H]^+$: 358.1449; found: 358.1465. HPLC purity: 98.6%.

2,3-Diethyl-4-fluoro-5-morpholinobenzo[de]chromene-7,8-dione (16b). 16b was prepared from K_2CO_3 (552 mg, 4 mmol), 12 (2 mmol), morpholine (6 mmol). Deep green solid, yield 36%. Mp: 315.4–317.2 °C. IR ν_{max} (KBr) cm⁻¹: 1631, 1695. ¹H NMR (400 MHz, CDCl_3) δ 7.75 (d, J = 7.6 Hz, 1H), 5.97 (s, 1H), 3.90 (d, J = 3.3 Hz, 4H), 3.26 (d, J = 2.9 Hz, 4H), 2.77–2.66 (m, 2H), 2.65–2.57 (m, 2H), 1.27 (t, J = 7.6 Hz, 3H), 1.20 (t, J = 7.1 Hz, 3H)¹³C NMR (101 MHz, CDCl_3 δ 179.7, 177.9, 165.5, 155.7, 153.7 (d, J = 263.6 Hz), 144.3 (d, J = 10.2 Hz), 126.7 (d, J = 4.02 Hz), 121.9 (d, J = 5.6 Hz), 121.5 (d, J= 11.9 Hz), 116.7 (d, J = 6.0 Hz), 111.2 (d, J = 4.4 Hz), 102.8, 66.7, 50.4, 23.8, 21.5 (d, J = 12.1 Hz), 15.1 (d, J = 4.4 Hz), 12.6. HRMS (ESI): calcd for $C_{20}H_{20}NO_4F[M + Na]^+$: 380.1269; found: 380.1254. HPLC purity: 99.8%.

5-(Diethylamino)-2,3-diethyl-4-fluorobenzo[de]chromene-7,8dione (16c). 16c was prepared from K₂CO₃ (552 mg, 4 mmol), 12 (2 mmol), diethylamine (6 mmol). Reddish black, yield 30%. Mp: 245.4–246.8 °C. IR $\nu_{\rm max}$ (KBr) cm⁻¹: 1624, 1694. ¹H NMR (400 MHz, CDCl_3) δ 7.67 (d, J = 7.9 Hz, 1H), 5.91 (s, 1H), 3.42 (m, 4H), 2.70 (m, 2H), 2.65–2.59 (m, 2H), 1.27 (t. J = 7.6 Hz, 3H), 1.21 (dd, J = 13.7, 6.8 Hz, 12H). 1.20 (t, J = 7.2 Hz, 3H)·¹³C NMR (101 MHz, CDCl_3) δ 180.6, 177.8, 166.2, 155.5, 151.2 (d, *J* = 259.1 Hz), 142.6 (d, J = 10.1 Hz), 126.7 (d, J = 2.8 Hz), 121.8 (d, J = 20.0 Hz), 121.8 (d, J = 7.8 Hz), 112.9 (d, J = 5.1 Hz), 111.59 (d, J = 4.5 Hz), 101.7, 46.4 (d, *J* = 5.2 Hz), 23.9, 21.6 (d, *J* = 12.6 Hz), 15.1 (d, *J* = 4.6 Hz), 13.3, 12.6. HRMS (ESI): calcd for C₂₀ H₂₂NO₃ F [M + H]⁺: 344.1656; found: 344.1671 HPLC purity: 99.8%.

2,3-Diethyl-4-fluoro-5-(pyrrolidin-1-yl)benzo[de]chromene-7,8dione (16d). 16d was prepared from K₂CO₃ (552 mg, 4 mmol), 12 (2 mmol), pyrrolidin (6 mmol). Dark green solid, yield 50%. Mp: 163.5–166.4 °C. IR ν_{max} (KBr) cm⁻¹: 1626, 1695. ¹H NMR (400 MHz, $CDCl_3$) δ 8.61 (d, J = 6.3 Hz, 1H), 8.00 (s, 1H), 7.76 (s, 1H), 6.50 (s, 1H), 6.01 (s, 1H), 2.67 (s, 2H), 2.58 (d, J = 7.1 Hz, 2H), 1.23 (d, J = 7.3 Hz, 4H), 1.18 (s, 7H). ¹³C NMR (101 MHz, CDCl₃) δ 180.8, 177.7, 166.5, 155.3, 148.4 (d, J = 256.0 Hz), 141.2 (d, J =11.3 Hz), 127.1 (d, J = 2.1 Hz), 121.2 (d, J = 12.3 Hz), 119.5 (d, J = 8.6 Hz), 111.6 (d, J = 4.2 Hz), 110.9 (d, J = 5.4 Hz), 101.1, 50.5, 25.4, 23.9, 21.7 (d, J = 21.7 Hz), 14.9 (d, J = 4.8 Hz), 12.55. HRMS (ESI): calcd for $C_{20}H_{20}NO_3F[M + H]^+$: 342.1500; found: 342.1511 HPLC purity: 99.6%.

9-Bromo-2,3-diethyl-4-fluoro-5-(piperidin-1-yl)benzo[de]chromene-7,8-dione (17). N-Bromosuccinimide (0.6 mmol, 1.2 equiv.) and benzoyl peroxide (12 mg) were added to a solution of 16a (0.5 mmol) dissolved in carbon tetrachloride. The reaction mixture was heated at 75 °C with stirring. After the starting material was consumed up, the reaction mixture was filtered and concentrated under reduced pressure. The solid residue was purified by using flash column chromatography (silica gel), eluting with dichloromethane to give 17 (56%) as black solid. Mp:307.9–310.1 °C. IR ν_{max} (KBr) cm⁻¹: 1636, 1689. ¹H NMR (400 MHz, CDCl_3) δ 7.79 (d, J = 7.7 Hz, 1H), 3.23 (m, J = 4.1 Hz, 4H), 2.71-2.76 (m, 4H), 1.77 (s, 4H), 1.66 (s, 2H), 1.35 (t, J = 7.4 Hz, 3H), 1.23 (t, J = 7.2 Hz, 3H). ¹³C NMR (101 MHz, CDCl3) δ 177.6, 172.5, 161.6, 155.7, 153.26 (d, J = 263.7 Hz), 145.2 (d, J = 9.8 Hz), 130.1, 128.4, 125.5 (d, J = 3.6 Hz), 123.2 (d, J = 6.3 Hz), 121.4 (d, *J* = 12.2 Hz), 115.0 (d, *J* = 5.8 Hz), 112.4 (d, *J* = 4.3 Hz), 97.6, 51.6, 29.8, 25.9, 24.0, 23.9, 21.6 (d, J = 12.7 Hz), 14.9 (d, J = 44 Hz), 12.1. HRMS (ESI): calcd for $C_{21}H_{21}NO_3FBr [M + H]^+$: 434.0762; found: 434.0770. HPLC purity: 98.3%.

MTT assay

Four different cancer cell lines, including HeLa, A549, K562 and HL-60 were used to evaluate the growth inhibitory effect of MsF derivatives by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-2H-tetrazolium-bromide) assay as described by Mosmann with modifications.23 The cells were plated at a density of 1 \times 10⁴ per well in 96-well microplates, and allowed to incubate overnight in a 5% CO2 incubator at 37 °C. MsF derivatives were added to the wells at increasing concentrations (0–50 μ M). After 48 h, each well was treated with 20 mL 2.5 mg mL⁻¹ MTT solution, and the cells were further incubated at 37 °C for 4 h. At the end of the incubation, the untransformed MTT was removed, and 100 µL of DMSO was added for HeLa and A549 cell lines. K562, and HL-60 cell lines were treated with three linked lysis solution, and then incubated at 37 °C overnight. The microplates were well shaken to dissolve the formazan dye, and the absorbance at 570 nm was measured using a microplate-reader (Bio-Tek). VP-16 were commercially available and used as positive controls.

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