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Synthesis and structure–activity relationships of thioflavone derivatives as specific inhibitors of the ERK-MAP kinase signaling pathway

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Abstract—Condensation of nitrobenzaldehydes **3** and α-[*o*-(*p*-methoxybenzylthio)benzoyl] sulfoxide **4** gave α-sulfinyl enones **5**. Treatment of **5** with formic acid caused cyclization followed by debenzylation to afford 3-(methylsulfinyl)thioflavanones **6**. Double-bond formation with elimination of methanesulfenic acid was performed by refluxing **6** in benzene, and, finally, the nitro group of 2-phenyl-4*H*-1-benzothiopyran-4-one (thioflavones) **7** was reduced with tin in tetrafluoroboric acid. Various 2′-aminothioflavones **8** thus prepared were evaluated for their inhibitory effects on the ERK-MAP kinase pathway. In a cell-based assay, 2-(2′-amino-3′-methoxyphenyl)-4*H*-1-benzothiopyran-4-one (**8b**) showed a more potent inhibitory effect than the corresponding oxygen compound (PD98059, **1**) on the Raf-induced activation of the ERK-MAP kinase pathway as well as cell proliferation. Furthermore, compound **8b** selectively and potently inhibited the proliferation of tumor cells in which the ERK-MAP kinase pathway is constitutively activated.

1. Introduction

The 41- and 43-kDa mitogen-activated protein (MAP) kinases (ERK2 and ERK1, respectively) play a pivotal role in the intracellular signaling pathway and are essential components of the ERK-MAP kinase signaling pathway, which includes MAP kinase/ERK kinase (MEK)1/2 and Raf-1.[1–3] The ERK-MAP kinase pathway is activated by a wide variety of mitogenic stimuli that interact with structurally distinct receptors, and it represents a convergence point for most, if not all, mitogenic signaling pathways. Very precise spatio-temporal control mechanisms for the ERK-MAP kinase activity have evolved to ensure homeostasis in multi-cellular organisms. Accordingly, aberrant activation of the ERK-MAP kinase pathway has been shown to be an essential feature common to many types of human tumors.[4–7] Thus, there has been an explosion of interest in the components of the ERK-MAP kinase pathway as attractive targets for the development of anticancer drugs.[8,9]

It has been shown that a flavone derivative, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059) (**1**), inhibits the MEK activity specifically and potently.[10,11] However, the structure–activity relationship of flavone derivatives related to **1** has not been reported. The isosteric replacement of an oxygen atom by a sulfur atom is useful for the design and synthesis of analogues, which are expected to display modified chemical and biological behavior.[12] In this respect, although differences in the physical and chemical behaviors between the flavone derivatives and the thioflavones have been well studied,[13,14] the difference in their biological activity has not been examined.

In this paper, we report the synthesis and structure–activity relationship of thioflavones bearing the poly-substituted phenyl group at the 2-position as inhibitors against the ERK-MAP kinase pathway.

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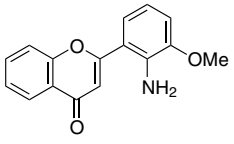
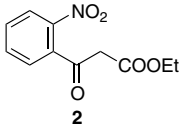
2. Chemistry

The synthetic procedure used for the preparation of **1**,[15,16] which has the flavone skeleton, cannot be applied to the synthesis of thioflavones because thioflavones have a structure in which a sulfur atom is replaced with an oxygen atom. Although many thioflavones have been prepared, a limited number of 2′-substituted ones are known. Two methods were selected from several reported procedures[17–23] for the synthesis of the 2′-substituted thioflavones: the direct condensation of a benzoylacetate with a benzenethiol[24–30] and the condensation of an α-[*o*-(alkylthio)benzoyl] sulfoxide with a benzenethiol followed by cyclization and elimination of a sulfenic acid.[31]

We first attempted to synthesize 2′-nitrothioflavone (**7a**) by condensation of ethyl *o*-nitrobenzoylacetate[32] (**2**) with benzenethiol using an acid, such as polyphosphoric acid, *p*-toluenesulfonic acid, or BF_3–Et_2O; all these trials, however, were unsuccessful. Therefore, a second approach was adopted.



1 (PD98059)



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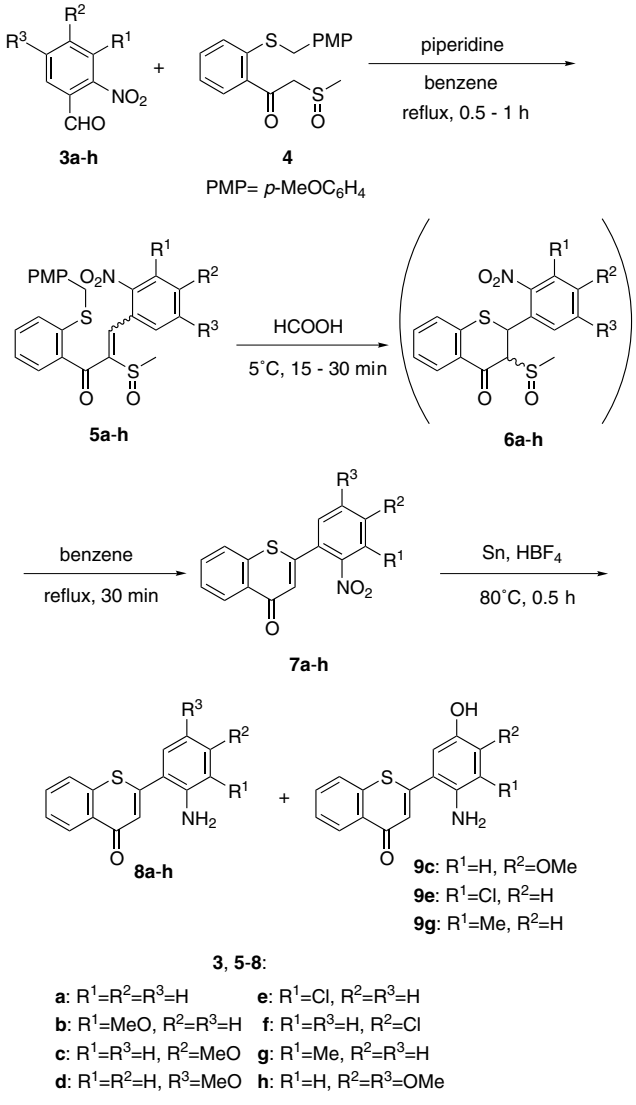
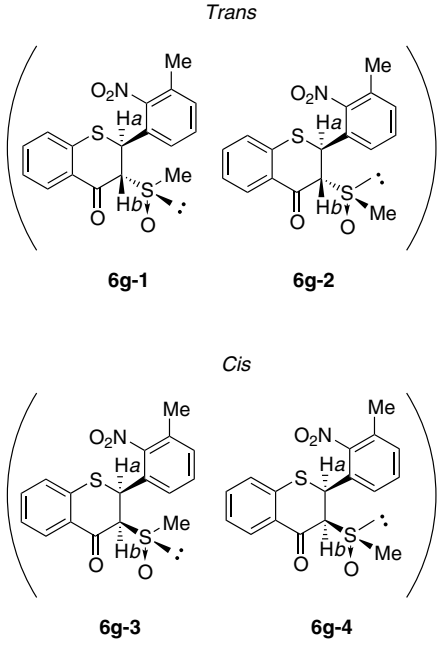
The 2′-nitrothioflavone derivatives were synthesized as shown in Scheme 1. *o*-Nitrobenzaldehydes **3** were prepared from *o*-nitroanilines or *o*-nitrobenzoic acids. Condensation of benzaldehydes **3** with α-benzoyl sulfoxide[31] **4** gave α-sulfinyl enones **5** in good yields. The ^1H NMR spectra of **5** showed the olefinic protons at δ 7.5–8.05 as singlets, but the geometrical structures of the double bonds could not be determined. Cyclization of **5** followed by debenzylation was performed by the treatment with formic acid at 5 °C to give 3-(methylsulfinyl)-2,3-dihydro-4*H*-1-benzothiopyran-4-ones **6** as a mixture of diastereoisomers. Refluxing the diastereomer mixtures of **6** in benzene caused the elimination of methanesulfenic acid to form thioflavones **7**.



Scheme 1.

Cyclized product **6g** as an example was separated into a *trans*-diastereoisomer and a mixture of two *cis*-isomers to examine the structures of **6**. The coupling constants between protons of the 2-position (H*a*) and the 3-position (H*b*) of three isomers were small and had similar values (J = 3–5 Hz) as shown in Figure 1. Therefore, their stereochemistry was determined by the NOE difference spectra: a compound showing a small NOE enhancement (5%) was a *trans*-isomer (**6g-1** or **6g-2**), and the others with a big enhancement (13% and 15%) were *cis*-isomers (**6g-3** and **6g-4**). The two *cis*-isomers would be the diastereoisomers due to the difference in stereochemistry between the sulfinyl group and the carbon atom at the 3-position, but their stereostructures have not been assigned yet. When a mixture of diastereoisomers of **6g** in chloroform was allowed to stand at room temperature, **6g** was gradually transformed into thioflavone **7g**.

A nitro group of **7** was reduced with tin and tetrafluoroboric acid to obtain 2′-aminothioflavones **8**. We used tetrafluoroboric acid instead of hydrochloric acid in order to prevent thioflavones **7** from chlorination of the aryl group at the 2-position. Reduction of 3′- or 4′-substituted 2′-nitrothioflavones **7c**,**e**,**g** gave 5′-hydroxylated products **9c**,**e**,**g** together with **8c**,**e**,**g**. The exact mass data (molecular formula: $C_{16}H_{13}NO_2S$) indicated that product **9c** contained one more oxygen than **8c**. The ^1H NMR spectrum of **9c** showed two singlets due to 3′- and 6′-protons at δ 6.34 and 6.90, respectively, and a new broad singlet due to a hydroxyl group that appeared at δ 5.20. An aromatic carbon without a substituent was reduced, and an aromatic carbon with a substituent was increased in the ^{13}C NMR spectrum. The IR spectrum exhibited an absorption at 3400 cm^{-1} due to the hydroxy group. The hydroxylation would proceed via the rear-



^{1}H-NMR (400MHz)

Isomers	H*a* (ppm)	H*b* (ppm)	SOMe (ppm)	*J* (Hz)
Trans	5.14	4.17	2.79	3
Cis-1	4.96	4.20	2.85	4
Cis-2	5.18	4.49	2.97	5

NOE measurement

	H*a*-H*b* (%)	H*b*-SOMe (%)
Trans	5	5
Cis-1	15	5
Cis-2	13	6

Figure 1.

rangement of an *N*-arylhydroxylamine formed in situ under acidic conditions (Bamberger rearrangement).[33,34]

3. Biological activity

Compounds **7a**–**g**, **8a**–**g,** and **9c**,**e**,**g** were examined for their ability to inhibit the Raf-1-induced activation of the ERK-MAP kinase pathway in a cell-based assay with the use of 3T3-ΔRaf-1:ER cells.[35,36] Among those studies, **8b** was found to have the most potent inhibitory activity on the activation of the ERK-MAP kinase pathway, which was more potent than that of **1**,[10,11] the corresponding oxygen compound of **8b** (IC_{50} of 1.88 μM vs 4.35 μM as determined by the phosphorylation of MEK1, and 2.95 μM vs 13.8 μM as determined by the phosphorylation of ERK1/2). Furthermore, **8b** inhibited the Raf-1-induced proliferation of the cells more potently than **1** (IC_{50} of 7.00 M vs 17.5 μM). Although **8c** also exhibited inhibitory activity, other compounds did not significantly inhibit the Raf-1-induced activation of ERK-MAP kinase pathway or cell proliferation at concentrations up to 100 μM (Table 1).

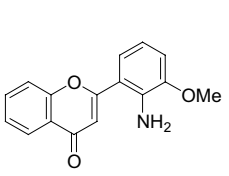
PD98059 (**1**) is a unique inhibitor of MEK1/2; it does not inhibit MEK1/2 activity directly but acts alike as an allosteric inhibitor, that is, it binds outside the ATP- and ERK1/2-binding sites on MEK1/2 and the resultant modification of the three-dimensional structures renders MEK1/2 not phosphorylatable (activatable) by Raf-1.[11] Likewise, **8b** did not inhibit the MEK1 activity directly as determined by the phosphorylation of ERK1/2 in an in vitro kinase assay, but it inhibited the phosphorylation of MEK1 by Raf-1 (Table 1). As in the case of the in vivo assay, **8b** exhibited a more potent inhibitory activity on the phosphorylation of MEK1 by Raf-1 than **1** (IC_{50} of 1.33 μM vs 7.55 μM). An assessment of other kinases, which include ERK1/2, p38 MAP kinase, c-Jun N-terminal kinase, MKK3, MAPKAP kinase 2, protein kinase Cα, EGF receptor kinase, and cyclin-dependent kinase 2, showed that none of these enzymes was significantly inhibited by **8b** or **8c** at concentrations as high as 100 μM. Furthermore, **8b** did not inhibit the phosphorylation of the myelin basic protein by Raf-1 in an in vitro assay (data not shown). These results indicate that substitution by sulfur of oxygen at the position 1 of the flavone structure, that is, conversion of flavone to thioflavone by itself, does not alter its specificity as an MEK inhibitor.

Finally, compound **8b** was tested for its ability to inhibit the proliferation of several tumor cell lines in culture. Compound **8b** inhibited the proliferation of tumor cells in which the ERK-MAP kinase pathway is constitutively activated (WiDr colon carcinoma cells and HT1080 fibrosarcoma cells),[6] but it did not inhibit significantly the proliferation of those in which constitutive activation of the ERK-MAP kinase pathway is not detected (PC3 prostate carcinoma cells and Colo320 colon carcinoma cells).[6] Furthermore, **8b** showed better inhibitory activity than **1** on the proliferation of tumor cells (IC_{50} of 8.6 μM vs 29.5 μM for HT1080 cells and IC_{50} of 10.0 μM vs 30.5 μM for WiDr cells), and totally inhibited their proliferation at 30–50 μM concentration. In contrast, the corresponding oxygen compound **1** at 50 μM, which is almost the highest concentration available in the culture medium, inhibited partly (70–80%) but not completely the proliferation of these tumor cells. One weak point of **1**, for all its high specificity, is the low solubility in aqueous medium. Conversion of flavone to thioflavone by itself did not resolve this point significantly: compound **8b**, for example, gave the maximum concentration of ~100 μM in the culture medium. However, more potent inhibitory activity conferred on thioflavone derivatives made **8b** readily possible to totally abolish the proliferation of tumor cells.

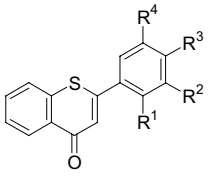
4. Structure–activity relationship

All the 2′-nitrothioflavones **7a**–**h** totally abolished the inhibitory activity, indicating that the amino group at the 2′-position is essential to exhibit the inhibitory effect on the activation of the ERK-MAP kinase pathway.

Table 1. Inhibitory activity of thioflavone derivatives



1 (PD98059)



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Compd	R^1	R^2	R^3	R^4	Cell-based assay [IC_{50} (μM)]			In vitro kinase assay [IC_{50} (μM)]	
					Phosphorylation of MEK1	Phosphorylation of ERK1/2	Cell Proliferation	Raf-1 → MEK1	MEK1 → ERK1/2
1	NH_2	OCH_3	H	H	4.35 ± 0.78	13.75 ± 3.18	17.50 ± 1.27	7.55 ± 1.48	>100
7a	NO_2	H	H	H	>100	>100	ND	>100	>100
7b	NO_2	OCH_3	H	H	>100	>100	ND	>100	>100
7c	NO_2	H	OCH_3	H	>100	>100	ND	>100	>100
7d	NO_2	H	H	OCH_3	>100	>100	ND	>100	>100
7e	NO_2	Cl	H	H	>100	>100	ND	>100	>100
7f	NO_2	H	Cl	H	>100	>100	ND	>100	>100
7g	NO_2	CH_3	H	H	>100	>100	ND	>100	>100
7h	NO_2	H	OCH_3	OCH_3	>100	>100	ND	>100	>100
8a	NH_2	H	H	H	>100	>100	ND	>100	>100
8b	NH_2	OCH_3	H	H	1.88 ± 0.11	2.95 ± 1.06	7.00 ± 0.57	1.33 ± 0.04	>100
8c	NH_2	H	OCH_3	H	21.50 ± 2.12	26.25 ± 1.06	22.85 ± 3.89	8.25 ± 3.18	>100
8d	NH_2	H	H	OCH_3	>100	>100	ND	>100	>100
8e	NH_2	Cl	H	H	>100	>100	ND	>100	>100
8f	NH_2	H	Cl	H	>100	>100	ND	>100	>100
8g	NH_2	CH_3	H	H	>100	>100	ND	>100	>100
8h	NH_2	H	OCH_3	OCH_3	>100	>100	ND	>100	>100
9c	NH_2	H	OCH_3	OH	>100	>100	ND	>100	>100
9e	NH_2	Cl	H	OH	>100	>100	ND	>100	>100
9g	NH_2	CH_3	H	OH	>100	>100	ND	>100	>100

Values are means ± SEM of three separate assays, each performed in duplicate.

The derivative bearing a methoxy group **8b** but not a methyl group **8g** at the 3′-position showed inhibitory activity, suggesting that an electron-donating group with lone-pair electrons at the 3′-position is necessary: the interaction, such as the hydrogen bond between the 3′-substituent and the binding site on the enzyme, would be important for revealing the inhibitory activity. The 4′-methoxy group decreased the activity, and compounds **8d** and **9c**,**e**,**g** bearing the 5′-methoxy or hydroxy group and the 4′,5′-dimethoxy derivative **8h** did not show the inhibitory activity. All these findings indicate that the methoxy group of the 2-phenyl group should be situated at the *ortho* position of the amino group.

The inhibitory activity of thioflavones against the activation of the ERK-MAP kinase pathway may depend largely on the polarizability of the chromone skeleton. The prominent activity observed for compound **8b** suggests that the replacement of 1-oxygen atom of the chromone skeleton with a sulfur atom promotes the polarization of the 4-carbonyl group,[13,14] which makes the binding of thioflavone **8b** to MEK easier and/or stronger than that of the flavone derivative **1**. This hypothesis would be supported by the fact that $MeSCH_2^+$ is more stable than $MeOCH_2^+$.[37] In contrast, a through-space effect of the methoxy group seems to be more important than its electronic effect because the thioflavone with the 3′-methoxy group showed the strongest activity.

5. Conclusion

We have prepared various derivatives of thioflavones **7**, **8**, and **9** and evaluated their inhibitory activity on the activation of the ERK-MAP kinase pathway. The 2-(2′-amino-3′-methoxyphenyl) derivative **8b** is the most potent compound among them and inhibits the Raf-1-induced activation of MEK more efficiently than the corresponding oxygen compound **1**. Furthermore, **8b** potently and selectively inhibits the proliferation of several tumor cells in which constitutive activation of the ERK-MAP kinase pathway is detected. These thioflavone derivatives would provide new leads for the development of potent and useful inhibitors against the ERK-MAP kinase pathway.

6. Experimental

6.1. Chemistry

6.1.1. 4-Methoxy-2-nitrobenzaldehyde (3c). This compound was prepared by the procedure for the synthesis

of 2-bromo-4-methylbenzaldehyde.[38] (a) A mixture of paraformaldehyde (3.67 g, 45 mmol) and hydroxylamine hydrochloride (3.16 g, 45 mmol) in water (5 mL) was warmed until the mixture became transparent. Then sodium acetate (3.71 g, 45 mmol) was added to the mixture. The whole mixture was gently heated under reflux for 15 min to give a 10% formaldoxime solution. (b) Concentrated hydrochloric acid (5.15 mL) was slowly added to a suspension of 4-methoxy-2-nitroaniline (5.0 g, 30 mmol) in water (5 mL), and then ice was added (9.0 g). The mixture was cooled and maintained at −5 to 5 °C in an ice–salt bath. A solution of sodium nitrite (2.05 g, 30 mmol) in water (3 mL) was added to the mixture with vigorous stirring, and the whole mixture was stirred for 15 min. The stirred solution of the diazonium salt was made neutral with a solution of sodium acetate (1.66 g, 20 mmol) in water (3 mL). (c) To the solution of formaldoxime prepared above (step a) were added copper(II) sulfate (712 mg, 4.5 mmol), sodium sulfite (120 mg, 0.95 mmol), and a solution of sodium acetate (19 g) in water (21 mL). The mixture was vigorously stirred at 10–15 °C, and the solution of the diazonium salt prepared in step b was gradually added to it. The whole mixture was stirred for 2 h at room temperature and then concentrated hydrochloric acid (27 mL) was added to it. The resulting mixture was gently refluxed for 2 h, cooled to room temperature, and then extracted with ether. The extracts were washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel eluting with CH_2Cl_2–AcOEt (30:1) to give red powder (1.43 g, 27%). The spectral data were identical with those of an authentic sample prepared by an alternative method.[39]

6.1.2. 5-Methoxy-2-nitrobenzaldehyde (3d). Thionyl chloride (4.53 g, 38 mmol) and DMF (2 drops) were added to a solution of 5-methoxy-2-nitrobenzoic acid (5 g, 25 mmol) in THF (25 mL), and the mixture was refluxed for 1 h and then cooled. The solution of 5-methoxy-2-nitrobenzoyl chloride thus prepared was added dropwise to a suspension of sodium borohydride (0.96 g, 25 mmol) in a mixture of DMF (12 mL) and THF (25 mL) at 0 °C. After the mixture was stirred for 3 h at room temperature, 3 N hydrochloric acid (25 mL) was added to it, and then the mixture was diluted with water (25 mL). The whole mixture was extracted with ether and the extracts were washed with brine and water, dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel eluting with hexane–AcOEt (2:1) to give 5-methoxy-2-nitrobenzyl alcohol (3.05 g, 78%). A solution of the benzylalcohol (3.05 g, 17 mmol) in CH_2Cl_2 (12 mL) was added to a solution of pyridinium chlorochromate (PCC) (5.33 g, 24 mmol) in CH_2Cl_2 (23 mL). The mixture was stirred for 2 h at room temperature and diluted with ether (75 mL). The organic layer was washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure to give yellow powder (2.65 g, 88%). The spectral data were identical with those of an authentic sample prepared by an alternative method.[40]

6.1.3. 3-Chloro-2-nitrobenzaldehyde (3e). This sample (0.85 g) was similarly prepared starting from 3-chloro-2-nitrobenzoic acid (1.75 g, 9 mmol) by the method for **3d**. The spectral data were identical with those of an authentic sample prepared by an alternative method.[41]

4-Chloro-2-nitrobenzaldehyde[41] (**3f**) and 3-methyl-2-nitrobenzaldehyde[42] (**3g**) were prepared by the known methods.

6.1.4. Methyl 2-(4-methoxybenylthio)benzoate.[31] Sodium hydride (60% pure in mineral oil) (7.36 g, 0.184 mol) was gradually added to a solution of methyl thiosalicylate[43] (31 g, 0.184 mol) in dry THF (300 mL). *p*-Methoxybenzyl chloride (25 mL, 0.184 mol) was added to the mixture of the thiosalicylate. The mixture was heated at 60 °C for 1 h and concentrated under reduced pressure. To the residue was added 3 N hydrochloric acid (100 mL), and the mixture was extracted with chloroform. The extracts were washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure. The oily product (47 g, 89%) was used for the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.78$ (3H, s, OMe), 3.89 (3H, s, OMe), 4.11 (2H, s, CH_2), 6.84 (2H, d, $J = 8$ Hz, Ar-H), 7.14 (1H, t, $J = 7$ Hz, Ar-H), 7.25–7.45 (4H, m, Ar-H), 7.95 (1H, d, $J = 8$ Hz, Ar-H).

6.1.5. 1-[2-(4-Methoxybenzylthio)phenyl]-2-(methylsulfinyl)ethanone (4).[31] Sodium methylsulfinylmethylide (dimsylsodium) was prepared from dry DMSO (162 mL) and sodium hydride (60% pure in mineral oil) (10.8 g, 0.27 mol) at 65–70 °C for 45 min under argon. Dry THF (80 mL) was added to the solution of dimsylsodium, and the mixture was cooled at 0 °C. A solution of methyl 2-(4-methoxybenylthio) benzoate (39 g, 0.135 mol) in dry THF (40 mL) was gradually added to the dimsylsodium solution at 0 °C under argon, and then the mixture was stirred for 2 h at room temperature. THF was evaporated under reduced pressure, and the residue was acidified by adding 3 N hydrochloric acid. The mixture was extracted with chloroform and the extracts were washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure. The residue was washed with ether, and then ether was removed in vacuo. The product (25.6 g, 57%) was used for the reaction with aldehydes without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.74$ (3H, s, SOMe), 3.78 (3H, s, OMe), 4.10 (2H, s, SCH_2Ar), 4.27 (1H, d, $J = 14$ Hz, $COCH_2SO$), 4.44 (1H, d, $J = 14$ Hz, $COCH_2SO$), 6.83 (2H, d, $J = 9$ Hz, Ar-H), 7.26 (2H, d, $J = 8$ Hz, Ar-H), 7.23–7.30 (1H, m, Ar-H), 7.42–7.51 (2H, m, Ar-H), 7.81 (1H, d, $J = 8$ Hz, Ar-H).

6.1.6. 1-[2-(4-Methoxybenzylthio)phenyl]-2-(methylsulfinyl)-3-(2-nitrophenyl)-2-propen-1-ones (5a). A mixture of α-sulfinyl ketone **4** (2 g, 6 mmol), *o*-nitrobenzaldehyde (0.90 g, 6 mmol), and piperidine (2 drops) in benzene (10 mL) was heated under reflux for 30 min and dehydrated using a Dean–Stark apparatus. Benzene was removed under reduced pressure, and the residue was

dissolved in chloroform. The solution was washed with 3 N hydrochloric acid and brine, dried ($MgSO_4$), and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel eluting with $CHCl_3$–AcOEt (10:1) to give yellow powder (1.84 g, 66%), mp 148 °C. IR (film): $\nu = 1063$ (SOMe), 1340 and 1522 (NO_2), 1617 (CO); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.86$ (3H, s, SOMe), 3.79 (3H, s, OMe), 4.02 (1H, d, $J = 12$ Hz, SCH_2-Ar), 4.05 (1H, d, $J = 12$ Hz, SCH_2-Ar), 6.83–6.88 (3H, m, Ar-H), 7.13–7.27 (8H, m, Ar-H), 7.48 (1H, d, $J = 8$ Hz, Ar-H), 7.96 (1H, d, $J = 8$ Hz, Ar-H), 8.05 (1H, s, C=CH); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 37.2$ (t), 40.7 (q), 55.3 (q), 114.1 (d), 124.4 (d), 124.8 (d), 127.3 (d), 127.9 (s), 129.7 (d), 130.1 (d), 130.4 (s), 130.5 (d), 131.5 (d), 132.5 (d), 133.3 (d), 135.6 (s), 136.9 (d), 140.5 (s), 146.4 (s), 147.0 (s), 159.0 (s), 192.3 (s); FABMS: m/z 468 (M^++1), 121 (base); elemental analysis calcd (%) for $C_{24}H_{21}NO_5S_2$: C, 61.65; H, 4.53; N, 3.00. Found: C, 61.60; H, 4.65; N, 3.00. The spectral data showed that product **5a** was a single isomer, but the geometrical structure was not determined.

6.1.7. 1-[2-(4-Methoxybenzylthio)phenyl]-2-(methylsulfinyl)-3-(3-methoxy-2-nitrophenyl)-2-propen-1-ones (5b). Yield 82%, yellow powder, mp 164 °C; IR (film): $\nu = 1064$ ($SOCH_3$), 1370 (NO_2), 1524 (NO_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.78$ (3H, s, $SOCH_3$), 3.80 (3H, s, Ar-OCH_3), 3.83 (3H, s, Ar-OCH_3), 4.09 (1H, d, $J = 14$ Hz, SCH_2), 4.13 (1H, d, $J = 14$ Hz, SCH_2), 6.68 (1H, d, $J = 8$ Hz, Ar-H), 6.83–6.88 (3H, m, Ar-H), 7.01–7.03 (2H, m, Ar-H), 7.29–7.33 (4H, m, Ar-H), 7.51 (1H, s, C=CH), 7.65 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 36.8$ (t), 40.6 (q), 55.3 (q), 56.5 (q), 113.5 (d), 114.1 (d), 121.8 (d), 124.2 (d), 126.5 (d), 127.8 (s), 127.9 (s), 130.1 (d), 130.5 (d), 131.0 (d), 132.1 (d), 133.1 (d), 133.8 (s), 139.5 (s), 142.2 (s), 150.1 (s), 151.1 (s), 159.0 (s), 192.0 (s); FABMS: m/z (relative intensity) 498 (M^++1, 7%), 121 (base); elemental analysis calcd (%) for $C_{25}H_{23}NO_6S_2$: C, 60.35; H, 4.66; N, 2.81. Found: C, 60.21; H, 4.63; N, 2.86.

6.1.8. 1-[2-(4-Methoxybenzylthio)phenyl]-2-(methylsulfinyl)-3-(4-methoxy-2-nitrophenyl)-2-propen-1-ones (5c). Yield 70%, yellow powder, mp 152–155 °C; IR (film): $\nu = 1075$ ($SOCH_3$), 1348 (NO_2), 1528 (NO_2), 1634 (CO); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.86$ (3H, s, $SOCH_3$), 3.77 (3H, s, Ar-OCH_3), 3.80 (3H, s, Ar-OCH_3), 4.05 (1H, d, $J = 12$ Hz, SCH_2), 4.08 (1H, d, $J = 12$ Hz, SCH_2), 6.72 (1H, d, $J = 8$ Hz, Ar-H), 6.85–6.89 (3H, m, Ar-H), 7.02 (1H, d, $J = 8$ Hz, Ar-H), 7.20–7.21 (2H, m, Ar-H), 7.26–7.28 (2H, m, Ar-H), 7.47 (1H, s, Ar-H), 7.52 (1H, d, $J = 8$ Hz, Ar-H), 7.96 (1H, s, C=CH); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 36.9$ (t), 40.8 (q), 55.3 (q), 55.9 (q), 109.4 (d), 114.1 (d), 119.69 (d), 122.2 (s), 124.2 (d), 127.0 (d), 127.9 (s), 130.1 (d), 130.8 (d), 132.6 (d), 132.7 (d), 135.2 (s), 136.5 (d), 140.8 (s), 145.8 (s), 147.3 (s), 159.0 (s), 160.3 (s), 192.5 (s); FABMS: m/z (relative intensity) 498 (M^++1, 7%), 154 (base); elemental analysis calcd (%) for $C_{25}H_{23}NO_6S_2$: C, 60.35; H, 4.66; N, 2.81. Found: C, 59.97; H, 4.61; N, 2.67.

6.1.9. 1-[2-(4-Methoxybenzylthio)phenyl]-2-(methylsulfinyl)-3-(5-methoxy-2-nitrophenyl)-2-propen-1-ones (5d). Yield 75%, yellow powder, mp 153–155 °C; IR (film): $\nu = 1071$ ($SOCH_3$), 1336 (NO_2), 1509 (NO_2), 1639 (CO); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.87$ (3H, s, $SOCH_3$), 3.79 (3H, s, Ar-OCH_3), 3.80 (3H, s, Ar-OCH_3), 3.97 (1H, d, $J = 12$ Hz, SCH_2), 4.07 (1H, d, $J = 12$ Hz, SCH_2), 6.58 (1H, d, $J = 3$ Hz, Ar-H), 6.74 (1H, dd, $J = 9$, 3 Hz, Ar-H), 6.85 (1H, d, $J = 8$ Hz, Ar-H), 6.91 (1H, dd, $J = 9$, 1 Hz, Ar-H), 7.15–7.20 (2H, m, Ar-H), 7.25 (2H, d, $J = 8$ Hz, Ar-H), 7.49 (1H, dd, $J = 8$, 1 Hz, Ar-H), 7.97 (1H, d, $J = 9$ Hz, Ar-H), 8.09 (1H, s, C=CH); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 37.2$ (q), 40.4 (t), 55.1 (q), 56.1 (q), 113.8 (d), 114.0 (d), 115.3 (d), 124.2 (d), 126.9 (d), 127.3 (d), 127.4 (s), 130.0 (d), 130.8 (d), 132.6 (d), 133.2 (s), 135.3 (s), 137.6 (d), 139.3 (s), 140.7 (s), 146.3 (s), 158.9 (s), 163.0 (s), 192.3 (s); FABMS: m/z (relative intensity) 498 (M^++1, 4%), 121 (base); elemental analysis calcd (%) for $C_{25}H_{23}NO_6S_2$: C, 60.34; H, 4.66; N, 2.81. Found: C, 59.93; H, 4.52; N, 2.79.

6.1.10. 1-[2-(*p*-Methoxybenzylthio)phenyl]-2-(methylsulfinyl)-3-(3-chloro-2-nitrophenyl)-2-propen-1-ones (5e). Yield 73%, yellow powder, mp 134–135 °C; IR (film): $\nu = 1074$ ($SOCH_3$), 1248 (NO_2), 1534 (NO_2), 1636 (CO); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.78$ (3H, s, $SOCH_3$), 3.80 (3H, s, Ar-OCH_3), 4.10 (1H, d, $J = 12$ Hz, SCH_2), 4.12 (1H, d, $J = 12$ Hz, SCH_2), 6.86 (2H, d, $J = 9$ Hz, Ar-H), 6.97 (1H, t, $J = 8$ Hz, Ar-H), 7.00–7.07 (2H, m, Ar-H), 7.26–7.34 (5H, m, Ar-H), 7.46 (1H, s, C=CH), 7.60 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 36.7$ (t), 40.5 (q), 55.3 (q), 114.1 (d), 124.3 (d), 125.7 (s), 126.5 (d), 127.7 (s), 128.3 (s), 128.9 (d), 129.4 (d), 130.1 (d), 130.5 (d), 131.2 (d), 132.0 (d), 133.4 (d), 133.5 (s), 142.2 (s), 147.5 (s), 151.6 (s), 159.0 (s), 191.6 (s); FABMS: m/z (relative intensity) 502 (M^++1, 35%), 121 (base); elemental analysis calcd for $C_{24}H_{20}ClNO_5S_2$: C, 57.42; H, 4.02; N, 2.79. Found: C, 57.23; H, 3.94; N, 2.87.

6.1.11. 1-[2-(*p*-Methoxybenzylthio)phenyl]-2-(methylsulfinyl)-3-(4-chloro-2-nitrophenyl)-2-propen-1-ones (5f). Yield 80%, yellow powder, mp 158 °C; IR (film): $\nu = 1073$ ($SOCH_3$), 1343 (NO_2), 1510 (NO_2), 1624 (CO); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.85$ (3H, s, $SOCH_3$), 3.80 (3H, s, Ar-OCH_3), 4.05 (1H, d, $J = 12$ Hz, SCH_2), 4.08 (1H, d, $J = 12$ Hz, SCH_2), 6.86 (2H, d, $J = 9$ Hz, Ar-H), 6.92 (1H, td, $J = 8$, 2 Hz, Ar-H), 7.05 (1H, d, $J = 8$ Hz, Ar-H), 7.12 (1H, dd, $J = 8$, 2 Hz, Ar-H), 7.23–7.28 (3H, m, Ar-H), 7.46 (1H, dd, $J = 8$, 1 Hz, Ar-H), 7.94 (1H, s, C=CH); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 36.9$ (t), 40.6 (q), 55.3 (q), 114.1 (d), 124.4 (d), 124.9 (d), 127.1 (d), 127.8 (s), 128.7 (s), 130.1 (d), 130.6 (d), 132.5 (d), 132.8 (d), 133.2 (d), 135.2 (s), 135.3 (d), 135.6 (s), 140.8 (s), 146.7 (s), 147.9 (s), 159.0 (s), 191.9 (s); FABMS m/z (relative intensity) 502 (M^++1, 25%), 154 (base); elemental analysis calcd (%) for $C_{24}H_{20}ClNO_5S_2$: C, 57.42; H, 4.02; N, 2.79. Found: C, 57.08; H, 3.95; N, 2.64.

6.1.12. 1-[2-(*p*-Methoxybenzylthio)phenyl]-2-(methylsulfinyl)-3-(3-methyl-2-nitrophenyl)-2-propen-1-ones (5g). Yield 69%, yellow powder, mp 157 °C; IR (film): $\nu = 1068$ ($SOCH_3$), 1246 (NO_2), 1523 (NO_2), 1628 (CO); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.30$ (3H, s, CH_3), 2.81 (3H, s, $SOCH_3$), 3.80 (3H, s, Ar-OCH_3), 4.08 (1H, d, $J = 12$ Hz, SCH_2), 4.11 (1H, d, $J = 12$ Hz, SCH_2), 6.86 (2H, d, $J = 8$ Hz, Ar-H), 6.96 (3H, m, Ar-H), 7.07 (1H, dd, $J = 9$, 3 Hz, Ar-H), 7.23–7.28 (2H, m, Ar-H), 7.30 (2H, d, $J = 9$ Hz, Ar-H), 7.58 (1H, s, C=CH), 7.63 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 18.0$ (q), 36.8 (t), 40.7 (q), 55.3 (q), 114.0 (d), 124.1 (d), 126.5 (d), 127.1 (s), 127.8 (s), 128.4 (d), 129.3 (s), 130.1 (d), 131.0 (d), 131.8 (s), 132.1 (d), 132.5 (d), 133.0 (d), 134.0 (d), 141.9 (s), 149.1 (s), 149.2 (s), 159.0 (s), 192.1 (s); FABMS: m/z (relative intensity) 482 (M^++1, 8%), 121 (base); elemental analysis calcd (%) for $C_{25}H_{23}NO_5S_2$: C, 62.35, H, 4.81, N, 2.91; found C, 62.06, H, 4.77, N, 2.85.

6.1.13. 1-[2-(4-Methoxybenzylthio)phenyl]-2-(methylsulfinyl)-3-(4,5-dimethoxy-2-nitrophenyl)-2-propen-1-ones (5h). Yield 61%, pale yellow powder, mp 181–182 °C; IR (film): $\nu = 1064$ ($SOCH_3$), 1324 (NO_2), 1520 (NO_2), 1635 (CO); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.87$ (3H, s, $SOCH_3$), 3.80 (3H, s, Ar-OCH_3), 3.86 (3H, s, Ar-OCH_3), 3.88 (3H, s, Ar-OCH_3), 3.95 (1H, d, $J = 12$ Hz, SCH_2), 4.09 (1H, d, $J = 12$ Hz, SCH_2), 6.50 (1H, s, Ar-H), 6.86 (2H, d, $J = 9$ Hz, Ar-H), 6.93 (1H, t, $J = 9$ Hz, Ar-H), 7.16–7.25 (4H, m, Ar-H), 7.54 (1H, s, Ar-H), 7.59 (1H, d, $J = 9$ Hz, Ar-H), 8.03 (1H, s, C=CH); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 36.9$ (t), 40.5 (q), 55.3 (q), 56.4 (q), 56.7 (q), 107.6 (d), 112.4 (d), 114.1 (d), 124.1 (d), 125.0 (s), 126.4 (d), 127.1 (d), 130.0 (d), 131.4 (d), 133.1 (d), 134.2 (s), 136.7 (d), 139.1 (s), 141.8 (s), 145.8 (s), 149.0 (s), 153.1 (s), 159.0 (s), 192.6 (s); FABMS: m/z (relative intensity) 528 (M^++1, 4%), 154 (base); elemental analysis calcd (%) for $C_{26}H_{25}NO_7S_2$: C, 59.19; H, 2.65; N, 4.78. Found: C, 59.07; H, 2.63; N, 4.69.

6.1.14. 2′-Nitrothioflavone (7a). Propenone **5a** (1.84 g, 3.9 mmol) was dissolved in 99% formic acid (4 mL) at 5 °C, and the solution was stirred for 30 min at that temperature. The mixture was made basic by adding sodium carbonate and then extracted with chloroform. The extracts were washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure to give a cyclized product **6a**. The raw product of **6a** was dissolved in benzene (10 mL), and the solution was refluxed for 30 min. The cooled organic layer was separated, and the residue was extracted with chloroform. The combined organic layers were washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel eluting with $CHCl_3$–AcOEt (10:1) to give white powder (602 mg, 54%), mp 142 °C. IR (film): $\nu = 1358$, 1528 (NO_2), 1622 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.92$ (1H, s, C=CH), 7.55–7.76 (6H, m, Ar-H), 8.08 (1H, d, $J = 8$ Hz, Ar-H), 8.54 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 114.0$ (d), 125.0 (d), 125.5 (d), 126.2 (d), 128.0 (d), 128.7 (d), 130.8 (s), 131.0 (d), 131.1 (s), 131.4 (d), 131.8 (d), 137.4 (s), 147.9 (s), 149.5 (s), 180.0 (s); MS: m/z (relative intensity) 283 (M^+, 42%), 136 (base); elemental analysis calcd (%) for $C_{15}H_9NO_3S$: C, 63.59; H, 3.29; N, 4.94. Found: C, 63.31; H, 3.21; N, 4.84.

6.1.15. 3′-Methoxy-2′-nitrothioflavone (7b). Yield 42%, white powder, mp 157 °C; IR (film): $\nu = 1375$ (NO_2), 1527 (NO_2), 1630 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.96$ (3H, s, OCH_3), 6.96 (1H, s, C=CH), 7.11 (1H, d, $J = 8$ Hz, Ar-H), 7.19 (1H, d, $J = 8$ Hz, Ar-H), 7.52–7.68 (4H, m, Ar-H), 8.49 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 56.7$ (q), 114.4 (d), 114.9 (d), 121.3 (d), 126.2 (d), 128.0 (d), 128.5 (d), 129.9 (s), 130.6 (s), 131.5 (d), 131.8 (d), 137.7 (s), 140.0 (s), 146.9 (s), 151.1 (s), 179.7 (s); MS: m/z (relative intensity) 313 (M^+, base); elemental analysis calcd (%) for $C_{16}H_{11}NO_4S$: C, 61.33; H, 3.54; N, 4.47. Found: C, 61.12; H, 3.66; N, 4.39.

6.1.16. 4′-Methoxy-2′-nitrothioflavone (7c). Yield 47%, white powder, mp 158 °C. IR (KBr): $\nu = 1333$, 1531 (NO_2), 1630 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.94$ (3H, s, OCH_3), 6.89 (1H, s, C=CH), 7.23 (1H, d, $J = 9$ Hz, Ar-H), 7.46 (1H, d, $J = 9$ Hz, Ar-H), 7.56–7.63 (4H, m, Ar-H), 8.54 (1H, d, $J = 8$ Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 56.1$ (q), 114.0 (d), 119.2 (d), 123.2 (s), 125.8 (d), 126.2 (d), 128.0 (d), 128.7 (d), 130.8 (s), 131.8 (d), 132.4 (d), 137.7 (s), 148.8 (s), 149.7 (s), 161.2 (s), 180.2 (s). MS: m/z (relative intensity) 313 (M^+, 42%), 136 (base); elemental analysis calcd (%) for $C_{16}H_{11}NO_4S$: C, 61.33; H, 3.54; N, 4.47. Found: C, 61.13; H, 3.77; N, 4.35.

6.1.17. 5′-Methoxy-2′-nitrothioflavone (7d). Yield 45%, white powder, mp 157 °C; IR (KBr): $\nu = 1357$ (NO_2), 1520 (NO_2), 1621 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.94$ (3H, s, OCH_3), 6.91 (1H, s, C=CH), 6.97 (1H, s, Ar-H), 7.08 (1H, d, $J = 9$ Hz, Ar-H), 7.55–7.64 (3H, m, Ar-H), 8.17 (1H, d, $J = 9$ Hz, Ar-H), 8.55 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 55.7$ (q), 114.9 (d), 116.2 (d), 124.6 (d), 125.8 (d), 127.3 (d), 127.4 (d), 128.2 (d), 130.3 (s), 131.3 (d), 133.3 (s), 137.0 (s), 140.0 (s), 149.8 (s), 152.7 (s), 179.5 (s); MS: m/z (relative intensity) 313 (M^+, base); elemental analysis calcd (%) for $C_{16}H_{11}NO_4S$: C, 61.33; H, 3.54; N, 4.47. Found: C, 61.05; H, 3.66; N, 4.45.

6.1.18. 3′-Chloro-2′-nitrothioflavone (7e). Yield 58%, yellow powder, mp 207 °C; IR (KBr): $\nu = 1360$, 1532 (NO_2), 1620 (C=O); ^{1}H NMR (400 MHz) ($CDCl_3$): $\delta = 6.95$ (1H, s, C=CH), 7.50 (1H, d, $J = 8$ Hz, Ar-H), 7.56–7.67 (5H, m, Ar-H), 8.52 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz) ($CDCl_3$): $\delta = 126.3$ (d), 126.8 (d), 127.3 (s), 128.3 (d), 128.7 (d), 128.8 (d), 128.2 (s), 130.6 (s), 130.7 (d), 131.3 (d), 132.1 (d), 132.3 (d), 137.1 (s), 145.9 (s), 148.1 (s), 179.6 (s); MS: m/z (relative intensity) 317 (M^+, 30%), 136 (base); elemental analysis calcd (%) for $C_{15}H_8ClNO_3S$: C, 56.70; H, 2.54; N, 4.17. Found: C, 56.44; H, 2.58; N, 4.37.

6.1.19. 4′-Chloro-2′-nitrothioflavone (7f). Yield 63%, yellow powder, mp 202 °C; IR (KBr): $\nu = 1334$ (NO_2), 1533 (NO_2), 1624 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.89$ (1H, s, C=CH), 7.52 (1H, d, $J = 8$ Hz, Ar-H), 7.57–7.67 (3H, m, Ar-H), 7.72 (1H, d, $J = 8$ Hz, Ar-H), 8.08 (1H, s, Ar-H), 8.54 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz), $CDCl_3$): $\delta = 125.3$ (d), 125.8 (d), 126.3 (d), 127.1 (s), 128.2 (d), 128.8 (d), 129.5 (d), 130.7 (d), 132.0 (d), 132.5 (d), 133.4 (s), 137.1 (s), 137.2 (s), 148.5 (s), 180.0 (s); MS: m/z (relative intensity) 317 (M^+, 40%), 136 (base); elemental analysis calcd (%) for $C_{15}H_8ClNO_3S$: C, 56.70; H, 2.54; N, 4.42. Found: C, 56.51; H, 2.58; N, 4.35.

6.1.20. 3′-Methyl-2′-nitrothioflavone (7g). Yield 45%, white powder, mp 178 °C; IR (KBr); $\nu = 1330$ (NO_2), 1520 (NO_2), 1624 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.43$ (3H, s, OCH_3), 6.94 (1H, s, C=CH), 7.37–7.67 (6H, m, Ar-H), 8.53 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 17.2$ (q), 125.7 (s), 125.8 (d), 127.5 (d), 127.6 (d), 128.2 (d), 128.7 (s), 130.0 (d), 130.3 (s), 130.6 (s), 131.3 (d), 132.7 (d), 137.0 (s), 147.2 (s), 149.2 (s), 179.2 (s). MS m/z: 297 (M^+, 45%), 136 (base); elemental analysis calcd (%) for $C_{16}H_{11}NO_3S$: C, 64.63; H, 3.73; N, 4.71. Found: C, 64.40; H, 3.88; N, 4.64.

6.1.21. 4′,5′-Dimethoxy-2′-nitrothioflavone (7h). Yield 51%, pale brown powder, mp 157–159 °C. IR (KBr): $\nu = 1331$, 1520 (NO_2), 1621 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.00$ (3H, s, OCH_3), 4.03 (3H, s, OCH_3), 6.90 (1H, s, C=CH), 6.93 (1H, s, Ar-H), 7.56–7.65 (3H, m, Ar-H), 7.70 (1H, s, Ar-H), 8.56 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 56.7$ (q), 56.8 (q), 108.1 (d), 112.9 (d), 125.4 (d), 126.2 (d), 127.9(d), 128.7 (d), 130.9 (s), 131.8 (d), 137.7 (s), 140.2 (s), 150.0 (s), 150.7 (s), 153.0 (s), 180.3 (s). MS: m/z (relative intensity) 343 (M^+, 55%), 179 (base); elemental analysis calcd (%) for $C_{17}H_{13}NO_5S$: C, 59.47; H, 3.82; N, 4.08. Found: C, 59.31; H, 4.00; N, 3.90.

6.1.22. 2′-Aminothioflavone (8a). 2′-Nitrothioflavone (**7a**) (592 mg, 2.1 mmol) was dissolved in hot tetrafluoroboric acid (42%) (5 mL), and tin (1.23 g, 10 mmol) was added to it. The mixture was stirred for 30 min at 80 °C, then cooled and made basic with an aqueous NaOH solution. The precipitate was collected by filtration and washed with chloroform. The washings were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel eluting with $CHCl_3$–AcOEt (10:1) to give yellow powder (200 mg, 38%), mp 131 °C. IR (KBr): $\nu = 1586$ (C=O), 3420 (NH_2), 3440 (NH_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.05$ (2H, br s, NH_2), 6.77–6.85 (2H, m, Ar-H), 7.11 (1H, s, C=CH), 7.23–7.27 (2H, m, Ar-H), 7.54–7.64 (3H, m, Ar-H), 8.56 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 116.3$ (d), 118.5 (d), 121.2 (s), 125.9 (d), 126.4 (d), 127.8 (d), 128.6 (d), 130.3 (d), 130.9 (s), 131.2 (d), 131.6 (d), 138.2 (s), 143.6 (s), 151.7 (s), 180.6 (s); MS: m/z (relative intensity) 253 (M^+, 43%), 236 (base); HRMS: calcd for $C_{15}H_{11}NOS$: 253.0561, found: 253.0556.

6.1.23. 2′-Amino-3′-methoxythioflavone (8b). Yield 89%, yellow powder, mp 194 °C; IR (KBr): $\nu = 1610$ (C=O), 3260 (NH_2), 3420 (NH_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.90$ (3H, s, OCH_3), 4.15 (2H, br s, NH_2), 6.78–6.91 (3H, m, Ar-H), 7.13 (1H, s, C=CH), 7.55–7.63 (3H, m, Ar-H), 8.55 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 55.8$ (q), 111.3 (d), 117.5 (d), 120.8 (s), 121.4 (d), 125.8 (d), 126.3 (d), 127.8 (d), 128.6 (d), 130.9 (s), 131.5 (d), 134.1 (s), 138.6 (s), 147.3 (s), 151.9 (s), 180.7 (s); MS: m/z (relative intensity) 283 (M^+, 60%), 266 (base); HRMS: calcd for $C_{15}H_{11}NOS$: 283.0667, found: 283.0666; elemental analysis calcd (%) for $C_{16}H_{13}NO_2S$: C, 67.82; H, 4.62; N, 4.94. Found: C, 67.62; H, 4.63; N, 4.80.

6.1.24. 2′-Amino-4′-methoxythioflavone (8c). Yield 64%, yellow powder, mp 150 °C; IR (KBr): $\nu = 1594$ (C=O), 3318 (NH_2), 3407 (NH_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.80$ (3H, s, OCH_3), 4.16 (2H, br s, NH_2), 6.30 (1H, s, Ar-H), 6.41 (1H, d, $J = 9$ Hz, Ar-H), 7.09 (1H, s, C=CH), 7.19 (1H, d, $J = 9$ Hz, Ar-H), 7.51–7.61 (3H, m, Ar-H), 8.53 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 55.5$ (q), 101.4 (d), 105.2 (d), 114.7 (s), 125.8 (d), 126.58 (d), 128.0 (d), 128.8 (d), 131.2 (s), 131.7 (d), 131.8 (d), 138.9 (s), 145.5 (s), 152.4 (s), 162.4 (s), 181.0 (s); MS: m/z (relative intensity) 283 (M^+, 50%), 266 (base); HRMS: calcd for $C_{16}H_{13}NO_2S$: 283.0667, found: 283.0655.

6.1.25. 2′-Amino-5′-hydroxy-4′-methoxythioflavone (9c). Yield 18%, yellow powder, mp 207 °C; IR (KBr): $\nu = 1610$ (C=O), 3340 (NH_2), 3415 (NH_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.25$ (2H, br s, NH_2), 3.95 (3H, s, OCH_3), 5.20 (1H, br s, OH), 6.34 (1H, s, Ar-H), 6.90 (1H, s, Ar-H), 7.10 (1H, s, C=CH), 7.54–7.63 (3H, m, Ar-H), 8.55 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 55.9$ (q), 100.0 (d), 113.6 (s), 115.2 (d), 125.7 (d), 126.3 (d), 127.7 (d), 128.6 (d), 130.9 (s), 131.5 (d), 137.5 (s), 138.6 (s), 138.7 (s), 149.1 (s), 151.9 (s), 180.8 (s); MS: m/z (relative intensity) 299 (M^+, 75%), 282 (base); HRMS: calcd for $C_{16}H_{13}NO_2S$: 299.0616, found: 299.0620.

6.1.26. 2′-Amino-5′-methoxythioflavone (8d). Yield 83%, yellow powder, mp 174 °C; IR (KBr): $\nu = 1620$ (C=O), 3380 (NH_2), 3420 (NH_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.78$ (3H, s, OCH_3), 4.25 (2H, br s, NH_2), 6.75 (1H, d, $J = 9$ Hz, Ar-H), 6.83 (1H, s, Ar-H), 6.88 (1H, d, $J = 9$ Hz, Ar-H), 7.11 (1H, s, C=CH), 7.55–7.59 (1H, m, Ar-H), 7.64 (2H, m, Ar-H), 8.56 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 55.9$ (q), 114.4 (d), 117.9 (d), 118.0 (d), 122.0 (s), 126.1 (d), 126.4 (d), 127.9 (d), 128.7 (d), 130.9 (s), 131.7 (d), 137.3 (s), 138.5 (s), 151.7 (s), 152.5 (s), 180.7 (s); MS: m/z (relative intensity) 283 (M^+, 48%), 266 (base); HRMS: calcd for $C_{16}H_{13}NO_2S$: 283.0667, found: 283.0670; elemental

analysis calcd (%) for $C_{16}H_{13}NO_2S$: C, 67.82; H, 4.62; N, 4.94. Found: C, 67.56; H, 4.61; N, 4.94.

6.1.27. 2′-Amino-3′-chlorothioflavone (8e). Yield 47%, white powder, mp 203 °C; IR (KBr): $\nu = 1600$ (C=O), 3200 (NH_2), 3260 (NH_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.50$ (2H, br s, NH_2), 6.78 (1H, t, $J = 8$ Hz, Ar-H), 7.09 (1H, s, C=CH), 7.16 (1H, d, $J = 8$ Hz, Ar-H), 7.36 (1H, d, $J = 8$ Hz, Ar-H), 7.54–7.59 (1H, m, Ar-H), 7.61–7.64 (2H, m, Ar-H), 8.54 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 126.4$ (d), 126.8 (d), 128.40 (d), 128.7 (d), 128.8 (d), 130.7 (s), 130.8 (s), 131.3 (d), 131.8 (s), 132.1 (d), 132.3 (d), 137.1 (s), 145.9 (s), 148.1 (s), 179.6 (s); MS: m/z (relative intensity) 287 (M^+, 85%), 270 (base); HRMS: calcd for $C_{15}H_{10}ClNOS$: 287.0172, found: 287.0177; elemental analysis calcd (%) for $C_{15}H_{10}ClNOS$: C, 62.61; H, 3.50; N, 4.87. Found: C, 62.42; H, 3.55; N, 4.88.

6.1.28. 2′-Amino-3′-chloro-5′-hydroxythioflavone (9e). Yield 32%, yellow powder, mp 239 °C; IR (KBr): $\nu = 1587$ (C=O), 3366 (NH_2), 3400 (OH), 2450 (NH_2); ^{1}H NMR (400 MHz, CD_3OD): $\delta = 6.71$ (1H, d, $J = 2$ Hz, Ar-H), 6.91 (1H, d, $J = 2$ Hz, Ar-H), 7.06 (1H, s, C=CH), 7.64 (1H, td, $J = 8$, 1 Hz, Ar-H), 7.74 (1H, td, $J = 8$, 1 Hz, Ar-H), 7.83 (1H, d, $J = 8$ Hz, Ar-H), 8.50 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 115.1$ (d), 117.9 (d), 119.3 (s), 122.1 (s), 125.9 (d), 126.8 (d), 127.6 (d), 127.9 (d), 130.3 (s), 131.8 (d), 134.0 (s), 137.9 (s), 148.2 (s), 150.2 (s), 179.4 (s); MS: m/z (relative intensity) 304 (M^++1, 45%), 154 (base); HRMS: calcd for $C_{15}H_{10}ClNO_2S$: 304.0199, found: 304.0206.

6.1.29. 2′-Amino-4′-chlorothioflavone (8f). Yield 57%, yellow powder, mp 180 °C; IR (KBr): $\nu = 1600$ (C=O), 3200 (NH_2), 3260 (NH_2). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.12$ (2H, br s, NH_2), 6.79 (1H, d, $J = 9$ Hz, Ar-H), 6.80 (1H, s, C=CH), 7.17 (1H, d, $J = 9$ Hz, Ar-H), 7.54–7.58 (1H, m, Ar-H), 7.61–7.63 (2H, m, Ar-H), 8.54 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 115.8$ (d), 118.5 (d), 119.5 (s), 126.1 (d), 126.3 (d), 128.0 (d), 128.7 (d), 130.9 (s), 131.0 (d), 131.7 (d), 137.0 (s), 138.3 (s), 144.7 (s), 150.7 (s), 180.5 (s). MS: m/z (relative intensity) 287 (M^+, 32%), 270 (base); HRMS: calcd for $C_{15}H_{10}ClNOS$: 287.0172, found: 287.0169.

6.1.30. 2′-Amino-3′-methylthioflavone (8g). Yield 26%, yellow powder, mp 161 °C; IR (KBr): $\nu = 1623$ (C=O), 3220 (NH_2), 3265 (NH_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.23$ (3H, s, CH_3), 4.25 (2H, br s, NH_2), 6.77 (1H, t, $J = 8$ Hz, Ar-H), 7.10 (1H, s, C=CH), 7.12–7.17 (2H, m, Ar-H), 7.54–7.57 (3H, m, Ar-H), 8.56 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 17.8$ (q), 118.0 (d), 121.1 (s), 123.2 (s), 126.1 (d), 126.4 (d), 127.6 (d), 127.8 (d), 128.6 (d), 130.9 (s), 131.6 (d), 132.2 (d), 138.7 (s), 141.7 (s), 152.4 (s), 180.7 (s); MS: m/z (relative intensity) 267 (M^+, 28%), 250 (base); HRMS: calcd for $C_{16}H_{13}NOS$: 267.0718, found: 267.0708; elemental analysis calcd (%) for $C_{16}H_{13}NOS$: C, 71.88; H, 4.90; N, 5.24. Found C, 71.68; H, 4.91; N, 5.17.

6.1.31. 2′-Amino-5′-hydroxy-3′-methylthioflavone (9g). Yield 26%, yellow powder, mp 203 °C; IR (KBr): $\nu = 1588$ (C=O), 3367 (OH). The absorption due to the amino group was overlapped with that of the hydroxy group; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.20$ (3H, s, CH_3), 3.65 (2H, br s, NH_2), 5.10 (1H, br s, OH), 6.64 (1H, s, Ar-H), 6.76 (1H, s, Ar-H), 7.07 (1H, s, C=CH), 7.57–7.63 (3H, m, Ar-H), 8.56 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 18.1$ (q), 113.8 (d), 120.0 (d), 121.9 (s), 125.1 (s), 126.1 (d), 126.4 (d), 127.9 (d), 128.7 (d), 130.9 (s), 131.7 (d), 135.3 (s), 138.6 (s), 147.8 (s), 152.3 (s), 180.8 (s); MS: m/z (relative intensity) 283 (M^+, 45%), 266 (base); HRMS: calcd for $C_{16}H_{13}NOS$: 283.0667, found: 283.0656.

6.1.32. 2′-Amino-4′,5′-dimethoxythioflavone (8h). Yield 39%, pale yellow powder, mp 170–171 °C; IR (KBr): $\nu = 1586$ (C=O), 3348 (NH_2), 3404 (NH_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.95$ (2H, br s, NH_2), 3.84 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 6.35 (1H, s, Ar-H), 6.81 (1H, s, Ar-H), 7.10 (1H, s, C=CH), 7.52–7.65 (3H, m, Ar-H), 8.54 (1H, d, $J = 8$ Hz, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 56.0$ (q), 56.6 (q), 100.8 (d), 112.5 (s), 113.2 (d), 125.7 (d), 126.3 (d), 127.8 (d), 128.6 (d), 130.9 (s), 131.6 (d), 138.5 (s), 142.3 (s), 151.8 (s), 152.1 (s), 180.8 (s); MS: m/z (relative intensity) 313 (M^+, 90%), 296 (base); HRMS: calcd for $C_{17}H_{15}NO_3S$: 313.0773, found: 313.0759.

6.2. Bioassays

Thioflavone derivatives were dissolved in DMSO to give 100 mM solutions and successive 10-fold dilutions were prepared. The test solution was added to each of culture media (cell-based assay) or reaction mixtures (in vitro kinase assay) to give the indicated final concentration: all the culture media/reaction mixtures received DMSO at a final concentration of 0.1%.

6.2.1. Cell-based assays. NIH3T3 cells expressing ΔRaf-1:ER (3T3-ΔRaf-1:ER)[35,36] were kindly provided by Dr. Martin McMahon (DNAX Research Institute) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. For the experimental use, sub-confluent cultures of 3T3-ΔRaf-1:ER cells in DMEM containing 10% fetal bovine serum were rendered quiescent by incubation for 24 h in serum-free medium (DMEM containing 2 mg/mL of bovine serum albumin, 1 μg/mL of insulin, 2 μg/mL of transferrin, 20 nM Na_2SeO_3, and 10 mM HEPES, pH 7.4) and pre-treated with respective thioflavone derivatives for 1 h. The cells were then stimulated with 1 μM β-estradiol to activate ΔRaf-1:ER for 2 h (for the analysis of Raf-1-induced activation of the ERK-MAP kinase pathway) or 48 h (for the analysis of Raf-1-induced cell proliferation). Raf-1-induced activation of the

ERK-MAP kinase pathway was analyzed by immunoblotting as described previously.[6,15,16] Briefly, the cells were scrapped off plates in a hypotonic cell lysis buffer (25 mM Tris–HCl, pH 7.4, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 25 mM glycerophosphate, 25 mM *p*-nitrophenyl phosphate, 20 nM okadaic acid, 0.2 mM sodium molybdate, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 1% aprotinin) and lysed by sonication for 60 s. Lysates were cleared by centrifugation at 12,000g for 30 min, and protein concentrations were determined using the BCA protein assay reagent (Pierce). Cell lysates (20 μg of protein) were separated by SDS-PAGE, electrophoretically transferred to an Immobilon-P membrane (Millipore Corp.), and subjected to immunodetection using the polyclonal anti-ERK1/2 antibody,[44,45] the monoclonal anti-phospho-ERK1/2 antibody (Sigma), the monoclonal anti-MEK1 antibody (Transduction Laboratories) or the polyclonal anti-phospho-MEK1/2 antibody (Cell Signaling) as the primary antibody and horseradish peroxidase-conjugated secondary antibody (Promega). Immunoreactive bands were visualized with the enhanced chemiluminescence system (Amersham Biosciences)[15,16] and were analyzed using a Fuji Luminescent image analyzer (LAS-1000 plus; Fuji Photo Film, Tokyo). For the analysis of Raf-1-induced cell proliferation, β-estradiol-stimulated 3T3-ΔRaf-1:ER cells were harvested by trypsinization and viable cells which excluded trypan blue were counted using a hemocytometer.

6.2.2. *In vitro* protein kinase assays. 3T3-ΔRaf-1:ER cells were stimulated with β-estradiol for 2 h. For assay of MEK, cell lysates prepared as described above (50 μg of protein) were immunoprecipitated by incubation for 3 h at 4 °C with the anti-MEK1 antibody pre-absorbed to Protein A-Sepharose.[15] After washing twice with the hypotonic cell lysis buffer and three times with MEK kinase buffer (50 mM Tris–HCl, pH 8.0, 0.5 mM EGTA, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol), each immunoprecipitate was treated for 30 min at 4 °C with respective thioflavone derivatives, and then incubated for 60 min at 30 °C with 20 μM ATP and 2.5 μg of GST-ERK2[KR] in 100 μL of MEK kinase buffer. After separating the reaction mixtures by SDS-PAGE, phosphorylation of GST-ERK2[KR] was analyzed by immunoblotting with the use of the monoclonal anti-phospho-ERK1/2 antibody as described above. The Raf-1 kinase activity was analyzed with a cascade assay that measured the phosphorylation of GST-ERK2[KR] in the presence of ΔRaf-1:ER and MEK1. In this assay, the MEK1-catalyzed phosphorylation of GST-ERK2[KR] was dependent on activation by Raf-1:ER.[10] In brief, β-estradiol-stimulated 3T3-ΔRaf-1:ER cells were lysed in R-cell lysis buffer (20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM sodium orthovanadate, and 1% aprotinin) on ice for 60 min. Cell lysates (100 μg of protein) were incubated with 2 μg of the polyclonal anti-ERα antibody (Santa Cruz Biotechnology) for 3 h at 4 °C. After collecting on protein-A Sepharose beads, immune complexes were washed twice with R-cell lysis buffer and three times with Raf-1 kinase buffer (30 mM Tris–HCl, pH 7.4, 7 mM $MnCl_2$, 5 mM $MgCl_2$, and 1 mM dithiothreitol), treated for 30 min at 4 °C with respective thioflavone derivatives, and then incubated for 60 min at 30 °C with 20 μM ATP and 5 μg of MEK1(FL) (Santa Cruz Biotechnology) in 100 μL of Raf-1 kinase buffer. After further incubation with 2.5 μg of GST-ERK2[KR] for 60 min at 30 °C, the extent of GST-ERK2[KR] phosphorylation was determined as described above. Assays of ERK1/2, p38 MAP kinase, c-Jun N-terminal kinase, MKK3, MAPKAP kinase 2, protein kinase Cα, EGF receptor kinase, and cyclin-dependent kinase 2, and phosphorylation of myelin basic protein by Raf-1 were performed as described previously.[6,15,16,46,47]

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