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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and bioevaluation of novel arylnaphthalene lignans as anticancer agents

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Abstract Novel arylnaphthalene lignans were synthesized and their structures were established by ^{1}H NMR, ^{13}C NMR, and HRMS. These compounds were evaluated for their in vitro cytotoxicity against cancer cell lines by MTT assay. Compound **5d** possessed the highest cytotoxicity against KB cells. Apoptosis of KB cells treated with **5d** was observed by acridine orange and ethidium bromide double staining assay. Western blot analysis disclosed that **5d** induced apoptosis via mitochondrial pathway accompanied by an increased expression of Bax and a decreased expression of Bcl-2.

Keywords Arylnaphthalene lignans · Synthesis · Cytotoxicity · Anticancer · Apoptosis

Introduction

There is an urgent need to develop novel agents for human cancer therapy (Jemal *et al*., 2005). Apoptosis is an important phenomenon in cancer chemotherapy, and the resistance to apoptosis is a hallmark of cancer cells. The crucial approach to anticancer drug discovery is selectively inducing apoptosis in cancer cells without causing excessive damage to normal cells (Fesik, 2005). A great number of anticancer drugs have been found to induce the apoptotic process in cancerous cells. Thus, discovery of more potent apoptosis regulators for cancer treatment is an attractive and prospective strategy (Huang, 2002).

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Arylnaphthalene lignans represent a significant subclass of lignans. Many of these arylnaphthalene lignans are derived from traditional herbs and have received increasing attention over the past decades for their interesting bioactivities (Foley *et al*., 2010); Fig. 1).

Diphyllin, an arylnaphthalene lignan isolated from many traditional medicinal plants, has been reported to possess anticancer and antiviral activities (Beers *et al*., 1988; Day *et al*., 2002). Previously, we have prepared a series of diphyllin derivatives and analogs (Zhao and Li, 2007; Zhao *et al*., 2008; Hui *et al*., 2012). Biologic assays disclosed that several compounds possessed potent anticancer activities and could induce apoptosis in various cancer cells. These results encouraged us to synthesize more compounds to fully discuss the SAR of diphyllin. In this paper, we report the synthesis, cytotoxicity, and apoptosis inducing activity of novel analogs of diphyllin (**5a–5j**, Scheme 1).

Results and discussion

Synthesis of arylnaphthalene lignans

The lignans **5a–5j** were synthesized as shown in Schemes 1 (Charlton *et al*., 1996; Ogiku *et al*., 1995). Compound **1** and ethylene–glycol were refluxed in benzene with *p*-TsOH·H_2O under a Dean-Stark trap to obtain acetal (**2**). The crude acetal **2** was dissolved in dry THF at −78 °C for half an hour and then warmed to room temperature for another 2.5 h to get **3a–3j**, which were used immediately in the following Diels–Alder reactions. They were heated with diethyl acetylenedicarboxylate (DEADC) and acetic acid at 140 °C for 1 h. The analogs (**5a–5j**) were prepared by one pot reduction and cyclization of **4a–4j** with $NaBH_4$ in methanol and subsequently with 10 % HCl (Scheme 1).

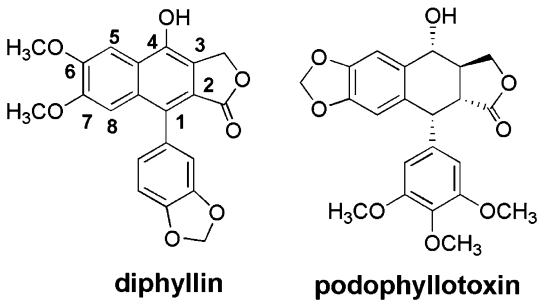


Fig. 1 Diphyllin and podophyllotoxin

Cytotoxicities of compounds **5a**–**5j** against cancer cells and HEK 293 cell

Compounds **5a**–**5j** were evaluated for their cytotoxic activities against three cancer cells and HEK 293 cell by a MTT growth inhibition assay. IC_{50} values were summarized in Table 1 and it represented the concentration inducing a 50 % decrease of cell growth after 3-days incubation.

In general, these derivatives displayed significant loss of cytotoxicities compared to diphyllin (**5a**). These results suggested that substitute pattern of D-ring was essential to their potency. Compound **5d** showed the best cytotoxicity among all the newly synthesized derivatives with the IC_{50} values of 5.1 μM in KB cell and 5.6 μM in SW480 cell. All these compounds did not possess apparent cytotoxicities against normal HEK 293 cell.

Induction of apoptosis in KB cells by compound **5d**

On the basis of the above results, further biologic evaluations have been focused on compound **5d**. KB cells were stained with AO/EB to verify the type of cell death.

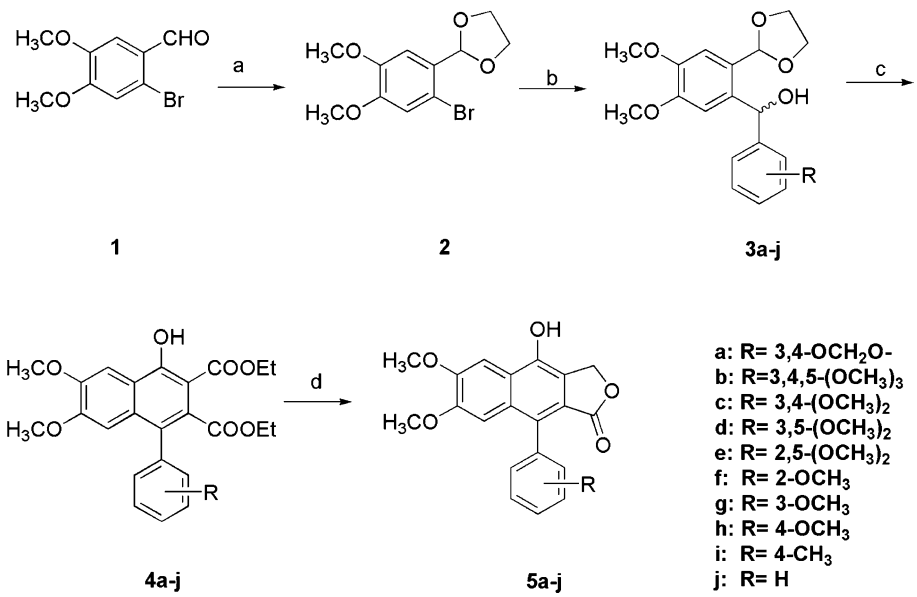
Table 1 Cytotoxicities against cancer cells and HEK 293 cell line of **5a**–**5j**

Compounds	IC_{50} (μM)				
	A549	SW480	KB	HCT-116	HEK 293
5a (diphyllin)	8.0	3.4	5.3	48.5	>100
5b	97.2	>100	23.2	>100	>100
5c	>100	>100	>100	>100	>100
5d	>100	5.6	5.1	29.3	>100
5e	>100	>100	>100	>100	>100
5f	>100	22.8	>100	>100	>100
5g	>100	>100	>100	88.5	>100
5h	>100	>100	20.6	>100	>100
5i	>100	>100	15.1	>100	>100
5j	>100	>100	>100	>100	>100
Etoposide	3.9	6.4	8.3	13.5	>100

The results obtained from the AO/EB staining are as shown in Fig. 2. Viable cells with intact DNA and nucleus showed homogeneous bright green. Early apoptotic cells showed green nuclei, but perinuclear chromatin condensation was visible as bright green patches or fragments. Late apoptotic cells' DNA was fragmented and stained orange. It was clear that most of the cells treated with compound **5d** exhibited typical characteristics of apoptotic cells like plasma membrane blebbing. This result also indicates that cell death occurred primarily through apoptosis.

Effect of compound **5d** on apoptosis-related proteins

Apoptosis is usually controlled by two major pathways—the mitochondrial pathway and membrane death receptor



Scheme 1 Synthesis of arylnaphthalene lignans. Reagents and conditions: *a* glycol, benzene, *p*-TsOH·H_2O, quant; *b* ArCHO, *n*-BuLi, THF, −78 °C, quant; *c* DEADC,CH_2Cl_2, AcOH, (70–75 %), *d* $NaBH_4$, MeOH, then 10 % HCl, 65 % (60–70 %)

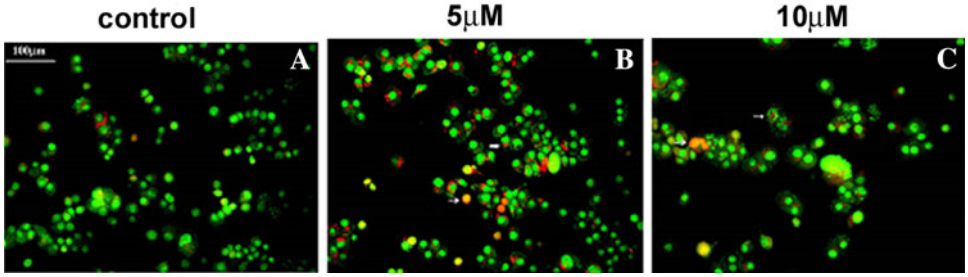


Fig. 2 Morphological study of KB cells treated with compound **5d** in 0, 5, and 10 μM for 72 h by AO/EB double staining. AO/EB (**A**–**C**) vacuolation and DNA fragmentation was obvious, *orange cells* indicate later apoptosis (Color figure online)

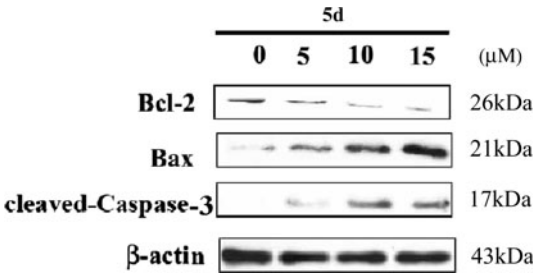


Fig. 3 Protein levels of cleaved-caspase-3, Bcl-2, and Bax in KB cells treated with compound **5d** were analyzed by Western blot. *β*-Actin was used normalize total proteins

pathway (Hegardt *et al*., 2001). To reveal the possible mechanisms responsible for the apoptotic effects of compound **5d**, we next evaluate its effects on the expression level of a series of proteins associated with apoptosis. Bcl-2 can prevent the release of cytochrome c from the mitochondria during apoptosis mediated by the mitochondrial pathway. In contrast, Bax can induce the release of cytochrome c from the mitochondria (Liu *et al*., 2006). The present results revealed that compound **5d** induced apoptosis via the mitochondrial pathway accompanied by an increased expression of Bax and a decreased expression of Bcl-2 (Fig. 3).

Conclusions

In summary a series of arylnaphthalene lignans have been synthesized by substituting D-ring of diphyllin with various aromatic rings. These analogs displayed moderate cytotoxicities against cancer cell lines. Apoptosis of KB cells induced by compound **5d** was observed by AO/EB staining assay and Western blot analysis. Compound **5d** induced apoptosis via the mitochondrial pathway accompanied by an increased expression of Bax and a decreased expression of Bcl-2.

Experimental

General methods

^{1}H NMR and ^{13}C NMR spectra were taken on a Bruker 300 MHz spectrometer with tetramethylsilane (TMS) as an internal standard and chemical shifts were recorded in values. The Mass spectra were obtained on a Shimadzu LCMS-2010EV (ESIMS).

General procedure for the synthesis of hydroxyl acetals

Acetal **2** was prepared according to literature. **2** (1.5 g, 5.4 mmol) was dissolved in dry THF (40 mL) under nitrogen, cooled to −78 °C, and *n*-BuLi (2.30 mL, 5.6 mmol) was added dropwise over 5 min. The mixture was stirred for another 15 min, followed by the dropwise addition of benzaldehyde (5.11 mmol) in THF (10 mL). After stirring for 30 min, the solution was gradually warmed to room temperature and stirred for another 2.5 h, followed by the addition of H_2O (30 mL). The resulting mixture was extracted with Et_2O (3× 30 mL), dried ($MgSO_4$), and concentrated to give a colorless solid (quant). These crude products were not further purified or characterized, but were used immediately in the following Diels–Alder reactions.

General procedure for the synthesis of arylnaphthalenes

Glacial acetic acid was added to a mixture of hydroxyacetal **3a**–**3j** (0.5 mmol) and DEADC (0.12 g, 0.7 mmol) in a minimum amount of CH_2Cl_2 and the temperature of the mixture was quickly brought up to 140 °C. The mixture was heated for 1 h. The cooled mixture was diluted with CH_2Cl_2 (10 mL), washed with 5 % sodium bicarbonate solution (3× 10 mL), dried ($MgSO_4$), and concentrated under vacuum. The residue was purified by flash chromatography (1:4, EtOAc-petroleum ether) to give arylnaphthalenes **4a**–**4j** (70–75 %).

*1-(3,4,5-Trimethoxyphenyl)-4-hydroxy-6,7-dimethoxy-naphthalene-2,3-dicarboxylate (**4b**)*

Yield 72 %, obtained as white solid: ^{1}H NMR($CDCl_3$): δ 12.43 (s, 1H, ArOH), 7.71 (s, 1H, ArH), 6.78 (s, 1H, ArH), 6.54 (s, 2H, ArH), 4.38 (dd, 2H, J = 11.4, 7.2 Hz, CH_2), 4.04 (s, 3H, OCH_3), 4.00 (dd, 2H, J = 14.4, 6.9 Hz, CH_2), 3.88 (s, 3H, OCH_3), 3.80 (s, 6H, OCH_3), 3.74 (s, 3H, OCH_3), 1.33 (t, 3H, J = 7.2 Hz, CH_3), 0.96 (t, 3H, J = 7.2 Hz, CH_3); ESI–MS: *m/z*, 537.5 [M+23].

*1-(3,4-Dimethoxyphenyl)-4-hydroxy-6,7-dimethoxy-naphthalene-2,3-dicarboxylate (**4c**)*

Yield 72 %, obtained as white solid: ^{1}H NMR ($CDCl_3$): δ 12.46 (s, 1H, ArOH), 7.74 (s, 1H, ArH), 6.96 (q, 3H, J = 7.8 Hz, ArH), 6.76 (s, 1H, ArH) 4.44 (q, 2H, J = 7.2 Hz, CH_2), 4.05 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3), 1.39 (t, 3H, J = 7.2 Hz, CH_3), 1.03 (t, 3H, J = 7.2 Hz, CH_3); ESI–MS: *m/z*, 507.4 [M+23].

*1-(3,5-Dimethoxyphenyl)-4-hydroxy-6,7-dimethoxy-naphthalene-2,3-dicarboxylate (**4d**)*

Yield 71 %, obtained as white solid: ^{1}H NMR ($CDCl_3$): δ 12.50 (s, 1H, ArH), 7.74 (s,1H, ArH), 6.82 (s, 1H, ArH), 6.51 (s, 3H, ArH), 4.45 (q, 2H, J = 7.2 Hz, CH_2), 3.79 (s, 6H, CH_3), 3.77 (s, 3H, CH_3), 1.39 (t, 3H, J = 7.2 Hz, CH_3); ESI–MS: *m/z*, 507.4 [M+23].

*1-(2,5-Dimethoxyphenyl)-4-hydroxy-6,7-dimethoxy-naphthalene-2,3-dicarboxylate (**4e**)*

Yield 73 %, obtained as white solid: ^{1}H NMR ($CDCl_3$): 12.47 (s, 1H, ArOH), 7.73 (s, 1H, ArH), 6.94 (s, 2H, ArH), 6.80 (s, 1H, ArH), 6.63 (s, 1H, ArH), 4.43 (m, 2H, CH_2), 4.07 (s, 3H, OCH_3), 3.99 (q, 2H, CH_2), 3.77 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3), 3.64 (s, 3H, OCH_3), 1.38 (t, 3H, J = 7.2 Hz, CH_3), 1.00 (s, 3H, J = 7.2 Hz, CH_3); ESI–MS: *m/z*, 507.4 [M+23].

*1-(2-Methoxyphenyl)-4-hydroxy-6,7-dimethoxy-naphthalene-2,3-dicarboxylate (**4f**)*

Yield 74 %, obtained as white solid: ^{1}H NMR ($CDCl_3$): δ 12.46 (s, 1H, ArOH), 7.77 (s, 1H, ArH), 7.40 (t, 1H, J = 8.1, ArH), 7.19 (d, 1H, J = 1.8 Hz, ArH), 7.05 (dd, 2H, J = 15.3 Hz, 7.2 Hz, ArH), 6.58 (s, 1H, ArH), 4.42 (m, 2H, CH_2), 4.04 (s, 3H, OCH_3), 4.00 (m, 2H, CH_2), 3.71 (s, 3H, OCH_3), 3.69 (s, 3H, OCH_3), 1.37 (t, 3H, J = 7.2 Hz, CH_3), 0.96 (t, 3H, J = 7.2 Hz, CH_3); ESI–MS: *m/z*, 477.4 [M+23].

*1-(3-Methoxyphenyl)-4-hydroxy-6,7-dimethoxy-naphthalene-2,3-dicarboxylate (**4g**)*

Yield 74 %, obtained as white solid: ^{1}H NMR($CDCl_3$): δ 12.42 (s, 1H, ArOH), 7.72 (s, 1H, ArH), 7.35 (q, 1H, J = 7.8 Hz, ArH), 6.94(q, 3H, J = 9.0 Hz, ArH), 6.72 (s, 1H, ArH), 4.42 (dd, 1H, J = 14.4, 7.2 Hz, CH_2), 4.03 (s, 3H, OCH_3), 3.97 (dd, 1H, J = 7.2 Hz, CH_2), 3.79 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 1.365 (t, 3H, J = 7.2 Hz, CH_3), 0.98 (t, 3H, J = 7.2 Hz, CH_3); ESI–MS: *m/z*, 477.4 [M+23].

*1-(4-Methoxyphenyl)-4-hydroxy-6,7-dimethoxy-naphthalene-2,3-dicarboxylate (**4h**)*

Yield 73 %, obtained as white solid: ^{1}H NMR($CDCl_3$): δ 12.42 (s, 1H, ArOH), 7.70 (s, 1H, ArH), 7.21 (d, 1H, J = 6.6 Hz, ArH), 6.94 (d, 1H, J = 9.0 Hz, ArH), 6.67 (s, 1H, ArH), 4.37 (dd, 2H, J = 14.4, 7.2 Hz, CH_2), 4.01 (s, 3H, OCH_3), 3.94 (dd, 2H, J = 14.1, 7.2 Hz, CH_2), 3.84 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 1.33 (t, 3H, J = 6.9 Hz, CH_3), 0.96 (t, 3H, J = 7.2 Hz, CH_3); ESI–MS: *m/z*, 477.4 [M+23].

*1-(4-Methylphenyl)-4-hydroxy-6,7-dimethoxy-naphthalene-2,3-dicarboxylate (**4i**)*

Yield 75 %, obtained as white solid: ^{1}H NMR ($CDCl_3$): δ 12.44 (s, 1H, ArOH), 7.75 (s, 1H, ArH), 7.27 (q, 4H, ArH), 6.72 (s, 1H, ArH), 4.42 (dd, 2H, J = 7.2 Hz, CH_2), 4.05 (s, 3H, OCH_3), 3.73 (dd, 2H, J = 7.2 Hz, CH_2), 2.43 (s, 3H, CH_3), 1.38 (t, 3H, J = 7.2 Hz,CH_3), 0.99 (t, 3H, J = 7.2 Hz, CH_3); ESI–MS: *m/z*, 461.4 [M+23].

*1-Phenyl-4-hydroxy-6,7-dimethoxy-naphthalene-2,3-dicarboxylate (**4j**)*

Yield 75 %, obtained as white solid: ^{1}H NMR ($CDCl_3$): 12.45 (s, 1H, ArH), 7.75 (s, 1H, ArH), 7.44 (d, 3H, J = 7.2 Hz, ArH), 7.34 (d, 2H, J = 7.8 Hz, ArH), 6.67 (s, 1H, ArH), 4.44 (dd, 2H, J = 6.9 Hz, CH_2), 4.05 (s, 3H, OCH_3), 3.99 (dd, 2H, J = 7.2 Hz, CH_2), 3.71 (s, 3H, OCH_3), 1.38 (t, 3H, J = 7.2 Hz, CH_3), 0.96 (t, 3H, J = 7.2 Hz, CH_3); ESI–MS: *m/z*, 447.3 [M+23].

General procedure for the synthesis of arylnaphthalene lignans

4a–**4j** (0.20 mmol), $NaBH_4$ (380 mg, 10 mmol), and MeOH (20 mL) were stirred under nitrogen at r.t. for 6 h. 10 % HCl (6 mL) was added cautiously and the mixture was stirred for 1 h, followed by the extraction with ether. The solution was concentrated to give a yellow solid which was then recrystallized from EtOH to afford pure **5a**–**5j** (65–72 %).

*1-(3,4,5-Trimethoxyphenyl)-4-hydroxy-3-(hydroxymethyl)-6,7-dimethoxynaphthalene-2-carboxylic Acid Lactone (**5b**)*

Yield 72 %, obtained as pale yellow solid: ^{1}H NMR (DMSO-d_6): δ 10.37 (s, 1H, ArOH), 7.59 (s, 1H, ArH), 7.01 (s, 1H, ArH), 6.58 (s, 2H, ArH), 5.32 (s, 2H, CH_2), 3.90 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 3.71 (s, 6H, 2× OCH_3), 3.63 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6): δ 170.0, 152.8, 152.7, 152.6, 151.0, 150.2, 145.3, 131.0, 130.4, 129.7, 122.2, 118.9,

108.7, 67.0, 60.6, 56.4, 56.3, 56.1, 55.6; HRMS calcd. for $C_{23}H_{23}O_8$ 427.1393, found 427.1388; 224 °C (dec).

*1-(3,4-Dimethoxyphenyl)-4-hydroxy-3-(hydroxymethyl)-6,7-dimethoxynaphthalene-2-carboxylic Acid Lactone (**5c**)*

Yield 69 %, obtained as pale yellow solid: ^{1}H NMR (DMSO-d_6): δ 7.62 (s, 1H, ArH), 7.05 (m, 2H, ArH), 6.89 (m, 2H, ArH), 5.35 (s, 2H, CH_2), 3.93 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 3.63 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6): 170.7, 150.9, 150.1, 148.6, 145.2, 130.5, 129.9, 127.8, 123.8, 123.3, 122.2, 119.0, 115.0, 111.6, 106.2, 101.2, 66.6, 60.2, 56.1, 56.0, 55.9, 55.6; HRMS calcd. for $C_{22}H_{20}O_7Na$ 419.1101, found 419.1112; 246 °C (dec).

*1-(3,5-Dimethoxyphenyl)-4-hydroxy-3-(hydroxymethyl)-6,7-dimethoxynaphthalene-2-carboxylic Acid Lactone (**5d**)*

Yield 70 %, obtained as pale yellow solid: ^{1}H NMR (DMSO-d_6): δ 10.40 (s, 1H, ArOH), 7.61 (s, 1H, ArH), 6.95 (s, 1H, ArH), 6.57 (s, 1H, ArH), 6.46 (s, 2H, ArH), 5.35 (s, 2H, CH_2), 3.93 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 3.63 (s, 3H, OCH_3), 3.34 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6): 169.9, 160.3, 150.9, 150.1, 145.4, 137.8, 130.1, 129.5, 123.6, 122.1, 118.9, 109.1, 105.9, 101.1, 100.0, 67.1, 60.2, 56.1, 56.0, 55.8, 55.6; HRMS calcd. for $C_{22}H_{20}O_7Na$ 419.1101, found 419.1121; 260 °C (dec).

*1-(2,5-Dimethoxyphenyl)-4-hydroxy-3-(hydroxymethyl)-6,7-dimethoxynaphthalene-2-carboxylic Acid Lactone (**5e**)*

Yield 68 %, obtained as pale yellow solid: ^{1}H NMR (DMSO-d_6): 7.61 (s, 1H, ArH), 7.05 (q, 2H, ArH), 6.77 (d, 2H, $J = 15.3$ Hz, ArH), 5.37 (s, 2H, CH_2), 3.93 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 3.60 (s, 3H, OCH_3), 3.53 (s, 3H, OCH_3), ^{13}C NMR (DMSO-d_6): 170.0, 153.2, 151.7, 150.9, 150.1, 145.4, 129.7, 126.7, 125.3, 123.7, 122.1, 119.5, 117. 8, 114.3, 112.6, 105.8, 101.1, 67.1, 56.2, 56.0, 55.8, 55.5; HRMS calcd. for $C_{22}H_{20}O_7Na$ 419.1101, found 419.1111; 216 °C (dec).

*1-(2-Methoxyphenyl)-4-hydroxy-3-(hydroxymethyl)-6,7-dimethoxynaphthalene-2-carboxylic Acid Lactone (**5f**)*

Yield 69 %, obtained as pale yellow solid: ^{1}H NMR (DMSO-d_6): 10.36 (s, 1H, ArOH), 7.62 (s, 1H, ArH), 7.46 (t, 1H, $J = 7.2$ Hz, ArH), 7.15 (t, 2H, $J = 7.2$ Hz, ArH), 7.06 (t, 1H, $J = 6.9$ Hz, ArH), 6.74 (s, 1H, ArH), 5.37 (s, 2H, CH_2), ^{13}C NMR (DMSO-d_6): 170.0, 157.6, 150.9, 150.0, 145.3, 132.0, 129.8, 126.9 124.3, 123.7, 122.1, 120.5, 119.6, 111.6, 105.7, 101.2, 100.0, 67.1, 56.0, 55.7, 55.4; HRMS calcd. for $C_{21}H_{18}O_6Na$ 389.0996, found 389.0990; 237 °C (dec).

*1-(3-Methoxyphenyl)-4-hydroxy-3-(hydroxymethyl)-6,7-dimethoxynaphthalene-2-carboxylic Acid Lactone (**5g**)*

Yield 71 %, obtained as pale yellow solid: ^{1}H NMR (DMSO-d_6): δ 7.62 (s, 1H, ArH), 7.41 (t, 1H, $J = 8.1$ Hz, ArH), 7.02 (d, 1H, $J = 8.1$ Hz, ArH), 6.90 (s, 1H, ArH), 6.87 (s, 2H, ArH), 5.35 (s, 2H, CH_2), 3.93 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 3.60 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6): δ 170.2, 159.0, 150.9, 150.1, 145.2, 132.9, 132.5, 130.4, 129.7, 127.5, 123.4, 122.4, 118.3, 113.8, 113.6, 105.5, 101.2, 67.0, 56.5, 55.8, 55.5; HRMS calcd. for $C_{21}H_{18}O_6Na$ 389.0996, found 389.0998; 238 °C (dec).

*1-(4-Methoxyphenyl)-4-hydroxy-3-(hydroxymethyl)-6,7-dimethoxynaphthalene-2-carboxylic Acid Lactone (**5h**)*

Yield 65 %, obtained as pale yellow solid: ^{1}H NMR (DMSO-d_6): δ 10.32 (s, 1H, ArOH), 7.58 (s, 1H, ArH), 7.19 (d, 2H, $J = 8.7$ Hz, ArH), 6.99 (d, 2H, $J = 8.7$ Hz, ArH), 6.89 (s, 1H, ArH), 5.32 (s, 2H, CH_2), 3.89 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 3.58 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6): δ 170.2, 159.0, 150.9, 150.1, 145.2, 132.1, 132.0, 130.3, 129.9, 127.5, 123.7, 122.2, 118.9, 113.7, 113.6, 105.9, 101.2, 67.0, 56.0, 55.5, 55.4; HRMS calcd. for $C_{21}H_{18}O_6Na$ 389.0996, found 389.0988; 218 °C (dec).

*1-(4-Methylphenyl)-4-hydroxy-3-(hydroxymethyl)-6,7-dimethoxynaphthalene-2-carboxylic Acid Lactone (**5i**)*

Yield 72 %, obtained as pale yellow solid: ^{1}H NMR (DMSO-d_6) 7.59 (s, 1H, ArH), 7.26 (m, 4H, ArH), 6.87 (s, 1H, ArH), 5.32 (s, 2H, CH_2), 3.90 (s, 3H, OCH_3), 3.36 (s, 3H, OCH_3), 2.37 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): 170.1, 150.9, 150.1, 145.3, 136.9, 132.6, 130.8, 130.5, 129.7, 128.9, 123.7, 122.2, 118.9, 105.9, 101.2, 100.0, 67.0, 60.2, 56.0, 55.5, 21.4; HRMS calcd. for $C_{21}H_{18}O_5Na$ 373.1046, found 373.1055; 244 °C (dec).

*1-Phenyl-4-hydroxy-3-(hydroxymethyl)-6,7-dimethoxynaphthalene-2-carboxylic Acid Lactone (**5j**)*

Yield 70 %, obtained as pale yellow solid: ^{1}H NMR (DMSO-d_6): 7.63 (s, 1H, ArH), 7.48 (s, 3H, ArH), 7.31 (d, 2H, $J = 8.7$ Hz, ArH), 6.85 (s, 1H, ArH), 5.371 (s, 2H, CH_2), 3.94 (s, 3H, OCH_3), 3.58 (s, 3H, OCH_3) ^{13}C NMR (DMSO-d_6): 170.1, 150.9, 150.1, 145.6, 135.7, 130.9, 130.3, 129.7, 128.3, 128.1, 127.9, 127.1, 123.7, 122.1, 118.9, 105.8 101.2, 67.1, 56.5, 56.0, 55.4; HRMS calcd. for $C_{20}H_{16}O_5Na$ 359.0890, found 359.0896; 239 °C (dec).

Cell culture

Four human cell lines, KB (oral squamous), A549 (lung), SW-480 (colon), and HEK 293 (renal) were cultured on RPMI-1640 medium supplemented with fetal bovine serum (10 %), penicillin (100U/mL), and streptomycin (100 μg/mL) in 25-cm^2 culture flasks at 37 °C in a humidified atmosphere with 5 % CO_2.

Cell viability

Cell viability was assessed by the MTT assay. Cells were harvested from the culture during the exponential growth phase, and seeded into multiwell culture plates at 5×10^4–1×10^5 cells/mL in fresh medium. After overnight growth, cells were treated with compounds (predissolved in DMSO) at selected concentrations for a period of 3 days. The medium was then discarded and replaced with MTT dye. Plates were incubated at 37 °C for 4 h. The resulting formazan crystals were solubilized in Lysis buffer (SDS 10 g, DMF 25 mL, H_2O 25 mL, acetic acid 1 mL, pH 4.7), and the optical density was read at 570 nm using a microplate reader (Biotek synergy 2).

Fluorescence morphological examination

Apoptotic morphology was studied by staining the cells with acridine orange (AO) and ethidium bromide (EB) (Srinivasan *et al*., 2007). Cells were washed three times with PBS after being incubated with compound **5d** in 0, 5, and 10 μM for 72 h, and were then stained with AO and EB for 3 min. Stained cells were viewed under a fluorescence microscope (Leica, German) with 200× magnification.

Western blot analysis

Cells were exposed to various concentrations of **5d** for 72 h. Cell lysates with identical amounts of protein were fractionated and transferred to PVDF membranes (Millipore Corporation, USA). The PVDF membrane was incubated in blocking buffer (TBS containing 0.1 % Tween 20 and 5 % nonfat milk) for 1 h at room temperature. Then, the membrane was incubated with the appropriate primary antibody overnight at 4 °C or 2 h at room temperature with gentle shaking. The membrane was washed thrice with rinsing buffer for 15 min and then incubated with the corresponding peroxidase conjugated secondary antibody for 1 h at room temperature. After repeating the washes in triplicate, the protein of interest was detected by enhanced chemiluminescence reagents from EC3 imaging system (UVP, USA).

Statistical analyses

Data were presented as Mean ± SE and analyzed by SPSS software. Picture were processed by means of Photoshop software. Mean values were obtained from at least three independent experiments.

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