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CA4

		R <sub>1</sub>	R <sub>2</sub>	IC <sub>50</sub> (CEM)
(CEM)	4n	3,4,5-(MeO) <sub>3</sub>	3'-OH-4'-MeO	1.7 nM
	4p	3,4,5-(MeO) <sub>3</sub>	3'-NH <sub>2</sub> -4'-MeO	1.4 nM

IC<sub>50</sub>=1.6 nM (CEM)

#### Synthesis and Antitumor Activity of Novel 3,4-diaryl Squaric Acid Analogues

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**Abstract:** A series of novel 3,4-diaryl squaric acid analogues **4a-r** related to combretastatin A-4 (CA4) using squaric acid as the *cis*-restricted linker were prepared and studied for their anticancer activity against selected human cancer cell lines. New compounds **4g**, **4k**, **4m**, **4n**, **4p**, **4q** and **4r** exhibit strong activities against human leukemia cells with IC<sub>50</sub> values of  $\leq 20$  nM and compounds **4k**, **4n**, **4p**, **4q** and **4r** showed potent activities against a panel of human tumor cell lines. Compounds **4n** and **4p** arrest tumor cell cycle in G2-M phase. Computational modeling analysis suggests that the binding mechanism of compound **4n** to the colchicine binding site on the microtubules is similar to that of CA4.

**Keywords**: 3,4-diaryl squaric acid analogues, anticancer, cytotoxic agents

#### **1. Introduction**

Microtubules are dynamic structures that play a crucial role in cell division, and are recognized as an important target for treating cancer [1-3]. Numerous chemically diverse antimitotic agents, many of which are derived from natural products, such as paclitaxel, epothilone A, vinblastine, combretastatin A-4 (CA4, Figure 1), and colchicine, can induce cancer cell apoptosis through interference with tubulin polymerization and depolymerization, and results in subsequent mitotic arrest. [4, 5]. Recently, it has been demonstrated that these microtubule depolymerizing anticancer compounds represented by CA4 can also function as vascular disrupting agents (VDA) that cause rapid and selective disruption of the established tumor vasculature [6, 7]. Since microtubule depolymerizing compounds can induce antivascular and antitumor effects at doses that are less than one-tenth of the maximum tolerated dose and have limited or nonexistent effects in normal tissue, the usefulness of compounds such as combretastatin A-4P (CA4P), in combination with cytotoxic chemotherapy and radiotherapy, is undergoing phase II trials, for the treatment of solid tumors. Another CA4 derivative, AVE-8062, is currently under clinical evaluation as a tumor vascular targeting agent (Fig.1). **ZD6126** which is a phosphate prodrug of N-acetyl colchinol, a derivative of colchicine, is now in phase II clinical trials [8-10].

**CA4**, a natural product isolated by Pettit et al. in 1989 from the South African willowtree *Combretum caffrum*, displayed potent cytotoxicity against a broad spectrum of human cancer cells including multiple drug-resistant cancer cell lines. The structural simplicity of **CA4** as well as its selective antivascular activity have

drawn significant attention to examine the structure-activity relationship (SAR) of this compound and its analogues [11-14]. SAR studies have demonstrated that the *cis* orientation of double bond and the presence of a 3,4,5-trimethoxyphenyl group are fundamental requirements for potent cytotoxitity. However, **CA4** is prone to isomerizes to the thermodynamically more stable and inactive *trans*-isomer, which significantly reduces its antiproliferative activities [15, 16]. Moreover, **CA4** as a clinical antitumor agent is limited by its low aqueous solubility and low bioavailability [17]. The goal to find even more potent and selective compounds has encouraged many researchers to design more soluble, stable and active analogs. In order to avoid the stability problems of **CA4**, suitable conformationally restricted rings such as heterocyclic rings were used in place of the ethene bridge [15-16, 18]. Many of these molecules have been evaluated for their effects on tubulin polymerization as well as for their antiproliferative activities, and possible mechanisms of action have also been investigated.



Fig. 1. Chemical structures of microtubule depolymerizing compounds.

We have earlier reported series of 3,4-diarylthiazol-2(3H)-ones (imines) [15],

1,5-diaryl-1*H*-pyrrole-2,3-diones [19], and 4-(diarylmethylene)piperidines [20] as potential anticancer agents against human cancer cell lines. In continuation of our efforts to search for novel anticancer agents, in this study we synthesized a variety of 3,4-diaryl squaric acid analogues using squaric acid as the *cis*-restricted linker and tested their anticancer activities against a selected panel of human cancer cell lines (Fig. 1). We also have investigated the influence of the position of the substituents with regard to both A- and B-aromatic rings, on the cytotoxicity activities of these analogues.

## 2. Results and discussion

#### 2.1. Chemistry

We have used three different methods to synthesize the 3,4-diaryl squaric acid

analogues. Compounds **4a-e** that contain electron-donating groups on the aromatic rings were prepared using sequential Friedel-Crafts procedures as outlined in Scheme 1. Squaric acid **1** in thionyl dichloride with DMF as catalyst was heated under reflux to afford 3,4-dichloro-3-cyclobutene-1,2-dione **2** [21] which on treatment with substituted benzenes under Friedel-Crafts conditions yielded 3-chloro-4-R<sub>2</sub>Ph-3-cyclobutene-1,2-dione **3**. Treatment of **3** with substituted benzenes under Friedel-Crafts conditions afforded the desired compounds **4a-e** in good yield.

Although derivatives **4f-k** also contained electron-donating groups on the aromatic ring, the Friedel-Crafts procedure was not successful using 1,2,3-trimethoxybenzene. As a result, we employed an alternate synthesis that is shown in Scheme 2. Derivatives **4f-k** was easily obtained under Stille cross-coupling [22] conditions by treating **3** with (3,4,5-trimethoxyphenyl)tri-n-butylstannane [23-24].



**Scheme 1.** Reagents and conditions: a. SOCl<sub>2</sub>, DMF, reflux, 3h; b. AlCl<sub>3</sub>, Ph-R<sub>2</sub>, 0°C, 1.5h; c. AlCl<sub>3</sub>, Ph-R<sub>1</sub>, 0°C, 1.5h



Scheme 2. Reagents and conditions: a. 3,4,5-(MeO)<sub>3</sub>-Ph-Sn(n-Bu)<sub>3</sub>, (CH<sub>3</sub>CN)<sub>2</sub>PdCl<sub>2</sub>, rt, 3h



Scheme 3. Reagents and conditions: a. Et<sub>3</sub>N, 4-OMe-Ph-SH, 0°C, 5h; b. 3,4,5-(MeO)<sub>3</sub>-Ph-

 $Sn(n-Bu)_3$ ,  $(CH_3CN)_2PdCl_2$ , rt, 3h; c. R<sub>2</sub>-Ar-B(OH)<sub>2</sub>, Pd<sub>2</sub>bda<sub>3</sub> (1%), TFP (3%) and CuTC, 50 $\Box$ , 20 h

For compounds **41-m**, **40**, and **4q-r** that contain electron-poor aromatic rings, both Stille cross-coupling [22] and Liebeskind-Srogl cross-coupling [22, 25] were used to prepare them (Scheme 3). 3-Chloro-4-(4-methoxyphenylthio)-3-cyclobutene-1,2-dione **5** was prepared in 90% yield by treating **2** with 0.5 equiv of *p*-methoxybenzenethiol with triethylamine as catalyst. Treatment of **5** with (3,4,5-trimethoxyphenyl)tri-*n*-butylstannane [23-24] under Stille cross-coupling conditions yielded the corresponding compound **6**. Reaction of **6** with various arylboronic acids [25-27] under Liebeskind-Srogl cross-coupling conditions gave the corresponding compounds **41-m**, **40**, **4q-r**. Compound **4n** was obtained from compound **4m** in the presence of NaHCO<sub>3</sub> in refluxing methanol [28]. Reduction of the nitro group of compound **4o** with Pd-C/H<sub>2</sub> in ethanol and EtOAc afforded the desired amino-substituted compound **4p**.

2.2. Biological Evaluations

**CA4**, delivered by its prodrug **CA4P**, is an important vascular disrupting agent. Our main objectives were to determine the cytotoxic activities and to attempt to gain some understanding of the antiproliferative mechanism of these new **CA4** analogues. Therefore, this series of 3,4-diaryl squaric acid analogues were evaluated for their *in vitro* anticancer activities against several cell lines and preliminary mode of action studies were performed for compound **4n**.

#### 2.2.1. In vitro cytotoxic activities and SAR

Since human CEM Leukemia cells are known for their rapid proliferation and high sensitivity to standard anticancer agents, the cytotoxic activities of the synthesized compounds were first evaluated against human CEM Leukemia cells (Table 1). Many compounds exhibited strong activities against human leukemia cells with IC<sub>50</sub> values of  $\leq 20$  nM (4g, 4k, 4m, 4n, 4p, 4q, 4r).

Similar to the well-known SAR of CA4, compound 4n, with a 3,4,5-trimethoxyphenyl ring and 3'- hydroxy-4'-methoxyphenyl ring, showed strong cytotoxic activity against human leukemia cells, as reflected by the IC<sub>50</sub> value of 1.7 nM, essentially the same as that for CA4 (IC<sub>50</sub> of 1.6 nM). This cytotoxic activity demonstrated that squaric acid ring system can be used as the *cis*-restricted linker in this series of compounds related to CA4.

In order to investigate the influence of the position of the substituents with regard to A- and B-aromatic rings and as the *p*-methoxy group in the B-ring of **CA4** is important for its activity, we first fixed the B-ring of this series of compounds with the 4'-methoxyphenyl ring and examined variations of the A-ring substituents. Comparing the A-ring derivatives **4a**, **4b**, **4c**, **4g**, the order of activity for the substituent(s) on the A-ring was 4-methoxy (**4a**) < 2,4-dimethoxy (**4b**), 3,4-dimethoxy (**4c**)< 3,4,5-trimethoxy (**4g**). For these four compounds, IC<sub>50</sub> values were >20 uM for **4a**, 1.51 uM for **4b** and 12.74 uM for **4c**, while for **4g** the IC<sub>50</sub> was 16 nM, much better than that of **4a**, **4b** and **4c**. These results are consistent with that found for **CA4** analogues, where it is well known that three methoxy groups on A ring are essential for potent cytotoxicity.

By keeping the 3,4,5-trimethoxyphenyl as a constant for the A-ring, we evaluated the antiproliferative effect of varying substitution on the B-ring for the squaric acid series. The number and position of methoxy substituents of the B-ring had a major influence on antiproliferative activity. The 3',4',5'-trimethoxy (4) derivative was the least active compound in the series, with an  $IC_{50}$  value of over 20  $\mu$ M. Replacement of the 3',4',5'- trimethoxy (4l) with the 3',4'-dimethoxy (4h) and 2',4'-dimethoxy (4i) resulted in increased activity. Replacement of the 3',4',5'trimethoxy (41) with the 4'-methoxy (4g) resulted in a marked increase in activity with  $IC_{50}$  value of 16 nM. However, replacement of the the 4'-methoxy(4g) with H (4f) resulted in significant loss of bioactivity. In an effort to increase potency, the 3'-hydroxy and 3'-amino substituted compounds **4n** and **4p** were prepared to mimic analogues of CA4. Both compounds 4n and 4p showed potent cytotoxic activity against human leukemia cells similar to that of CA4. The insertion of a 3'-chloro substituent on the B ring to yield 4j gave a 10-fold less potent compound than that of 4g. However, the insertion of a 3'-fluoro substituent on the B ring to yield 4k maintained the cytotoxicity compared with compound 4g. The indole compounds 4qand 4r, exhibited considerable cytotoxicity against human leukemia cells with  $IC_{50}$ values of 15.4 nM and 6.6 nM, respectively. This result suggested that the indole core is well tolerated as a replacement of the 4-methoxyphenyl B ring of CA4 with the retention of potent cytotoxic activity.

#### Table 1

Cytotoxicity of synthesized compounds and CA4 in CEM leukemia cells



Compound	R <sub>1</sub>	$R_2$	Cytotoxicity
			IC <sub>50</sub> , uM <sup>a</sup>
<b>4</b> a	4-MeO	4'-MeO	>20
<b>4</b> b	2,4-(MeO) <sub>2</sub>	4'-MeO	1.51
<b>4</b> c	3,4-(MeO) <sub>2</sub>	4'-MeO	12.74
<b>4d</b>	2,4,6-(MeO) <sub>3</sub>	4'-MeO	0.85
<b>4e</b>	2,3,4-(MeO) <sub>3</sub>	4'-MeO	2.48
<b>4f</b>	3,4,5-(MeO) <sub>3</sub>	Н	16.1
<b>4</b> g	3,4,5-(MeO) <sub>3</sub>	4'-MeO	0.016
<b>4h</b>	3,4,5-(MeO) <sub>3</sub>	3',4'-(MeO) <sub>2</sub>	0.21
<b>4i</b>	3,4,5-(MeO) <sub>3</sub>	2',4'-(MeO) <sub>2</sub>	1.77
<b>4</b> j	3,4,5-(MeO) <sub>3</sub>	3'-Cl-4'-MeO	0.136
<b>4</b> k	3,4,5-(MeO) <sub>3</sub>	3'-F-4'-MeO	0.0115
41	3,4,5-(MeO) <sub>3</sub>	3',4',5'-(MeO) <sub>3</sub>	>20
<b>4m</b>	3,4,5-(MeO) <sub>3</sub>	3'-AcO-4'-MeO	0.0018
<b>4n</b>	3,4,5-(MeO) <sub>3</sub>	3'-OH-4'-MeO	0.0017
40	3,4,5-(MeO) <sub>3</sub>	3'-NO <sub>2</sub> -4'-MeO	12.17
4p	3,4,5-(MeO) <sub>3</sub>	3'-NH <sub>2</sub> -4'-MeO	0.0014
<b>4</b> q	3,4,5-(MeO) <sub>3</sub>	1H-indol-5'-substituted	0.0154
	$\square$	composed by $R_2$ and the	
		benzene ring	
<b>4r</b>	3,4,5-(MeO) <sub>3</sub>	1H-indol-6'-substituted	0.0066
		composed by $R_2$ and the	
		benzene ring	
CA4			0.0016

 ${}^{a}IC_{50}$  values, concentration required to inhibit 50% of human tumor cells proliferation after 72 h treatment.

2.2.2. In vitro anticancer activity of 4k, 4n, 4p, 4q and 4r against several cancer cell lines

Since compounds **4k**, **4n**, **4p**, **4q** and **4r** showed potent activity against the non-solid human CEM cell line, they were further evaluated against a panel of human tumor cell lines including human liver cancer cells Bel-7402, HepG2, SMMC-7221, human breast cancer cells MCF-7, human pancreatic cancer cells SW-1990, human colon adenocarcinoma HCT116 and human leukemia cells CEM with CA4 employed as a positive control (Table 2).

Compounds **4n** and **4p** showed potent cytotoxicity against almost all seven cell lines studied. The highest cytotoxicity (IC<sub>50</sub> < 2 nM for both compound **4n** and **4p**) was observed against leukemia cells. The least sensitive cell line for compound **4n** was human liver cancer cells SMMC-7221, and for compound **4p** was human pancreatic cancer cells SW-1990. For compound **4n** and **4p**, the cytotoxicity of human liver cancer cell HepG2 were also high, with IC<sub>50</sub> values of less than 14 nM, similar to the positive control **CA4**. Moreover, the cytotoxicity of compound **4n** against human liver cancer cells Bel-7402 and human breast cancer cells MCF-7 was 5- to 6-fold stronger than that of compound **CA4**, and the cytotoxicity of compound **4p** against human liver cancer cells Bel-7402 was 122-fold stronger than that of compound **CA4**. However, the cytotoxicity of compound **4p** against human breast cancer cells MCF-7 and human pancreatic cancer cells SW-1990 were 5- to 7-fold less active than that of compound **CA4**.

With the goal of obtaining more SAR information, we also examined the antiproliferative activities of compounds **4q**, **4r** and **4k** compared to compound **4n** against 7 cancer cell lines. From these results, it can be seen that antiproliferative activities of the indole compounds **4q** and **4r** against leukemia cells and human liver cancer cells HepG2, are quite similar to that of **4n**. However, the cytotoxicities of compounds **4q** and **4r** against human liver cancer cells Bel-7402 were 5- to 12-fold less potent than compound **4n**. Interestingly, introduction of a 3'-fluoro substituent on the B ring (**4k**) displayed strong growth inhibitory activity, with IC<sub>50</sub> values lower than 200 nM against all 7 cell lines. These SAR results further demonstrate that B-ring was amenable to modifications to yield potent compounds and the 3'-hydroxy substituent of the B-ring is not indispensable for potent biological activities in this 3,4-diaryl squaric acid analogues series.

#### Table 2

The I	$C_{50}$ values	(µM) of	compounds	4n, 4p,	4q, 4r,	, <b>4k</b> , and	CA4	versus	various	cancer	cell	lines.
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Cell line	$\mathrm{IC}_{50},\mu\mathrm{M}^{\mathrm{a}}$								
¥.	4n	<b>4</b> p	<b>4</b> q	4r	<b>4</b> k	CA4			
CEM	0.0017	0.0014	0.0154	0.0066	0.0115	0.0016			
Bel-7402	0.309	0.014	1.496	3.621	0.013	1.718			
HepG2	0.014	0.005	0.006	0.006	0.105	0.006			
SMMC-7721	1.517	0.190	0.646	2.544	0.176	0.289			

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MCF-7	0.006	0.164	NT <sup>b</sup>	$NT^{b}$	0.005	0.029
SW-1990	0.245	1.768	NT <sup>b</sup>	$NT^{b}$	0.027	0.250
HCT116	0.005	0.005	$\mathrm{NT}^{\mathrm{b}}$	$NT^{b}$	0.081	0.006

 ${}^{a}IC_{50}$  values, concentration required to inhibit 50% of human tumor cells proliferation after 72 h treatment.

<sup>b</sup>NT = not tested

2.2.3. Mode of action of 4n

Compound **4n** has shown potent *in vitro* activities, and initiation of mode of action studies seemed warranted. Flow cytometric analysis with MCF-7 cells (Figure 3) was performed using **CA4** as the positive control. The results suggest that the compound **4n** blocks the cell cycle in the G2-M phase.



**Fig. 2.** Cell cycle analysis. MCF-7 cells untreated or treated with **CA4** and **4n** were analyzed for cell cycle distribution using flow cytometry.

#### 2.2.4. Molecular modeling study

To further expand the understanding of our experimental findings, molecular modeling studies for **4n** and **CA4** were carried out with the use of CDOCKER within the Discovery Studio 3.0 program package. Compounds **4n** and **CA4** were docked into the colchicine binding site of tubulin (PDB 1SA0). The binding mode observed of **4n** was similar to that of **CA4**. The trimethoxyphenyl group ring occupied the same position as the corresponding moiety of **CA4** and showed nonpolar interactions with Cys241, Leu248, Ala250 and Leu255. The B-rings of both compounds also occupied the same pocket, established one hydrogen-bond interaction with Met259 and made hydrophobic interactions with Ala180, Met259 and Lys352. Thus, results of the computational modeling analysis are consistent with our experimental studies (Fig. 3).



**Fig. 3.** Docking conformation of **4n** (yellow) and **CA4** (cyan) in the colchicine binding site of tubulin (PDB 1SA0) using CDOCKER program in Discovery Studio 3.0.

## 3. Conclusion

In this study, synthetic and activity studies for the 3,4-diaryl squaric acid analogues as a new class of potent anticancer agents were performed. New compounds 4g, 4k, 4m, 4n, 4p, 4q and 4r exhibit strong activities against human leukemia cells and compounds 4k, 4n, 4p, 4q and 4r showed potent activities against a panel of human tumor cell lines. This cytotoxic activity showed that the squaric acid ring system can be used for the *cis*-restricted linker in this series of compounds related to CA4. The SAR information revealed that all three methoxy groups of A ring are essential for potent cytotoxicity. Replacement of the B ring of CA4 with the indole core and replacement of the 3'-hydroxy substituent of the B-ring with 3'-amino or 3'-fluoro is tolerated with retention of the potent cytotoxic activity. Preliminary mode of action studies demonstrated that compound 4n arrests the tumor cell cycle in the G2-M phase. All these results are important for the design of structurally-related tubulin inhibitors or combretastatin analogs in the future.

#### 4. Experimental section

4.1. Chemistry Melting points were recorded using a Mel-Temp 3.0 capillary melting point apparatus and are uncorrected. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded employing a Bruker 400 Ultrashield<sup>TM</sup> instrument; chemical shifts ( $\delta$ ) are in ppm relative to internal TMS. Mass spectra were recorded on a VG analytical 70-SE spectrometer. Elemental analyses were obtained from Atlantic Microlab, Inc. (Norcross, GA).

## 4.1.1. 3,4-bis(4-methoxyphenyl)cyclobut-3-ene-1,2-dione (4a)

A mixture of squaric acid (0.55 g, 4.8 mmol), thionyl dichloride (0.88 ml, 9.7 mmol) and DMF (5 drops) was heated at reflux for 3 h. The resultant mixture was

cooled to 0°C. Anhydrous methylene chloride (6 ml) and anhydrous AlCl<sub>3</sub> (0.64 g, 4.8 mmol) were added to the reaction mixture. Then anisole (0.52 g, 4.8 mmol) was added to the mixture dropwise. The mixture was stirred at 0°C for 1.5 h. The resultant mixture was poured onto crushed ice and extracted with  $CH_2Cl_2$  (2×15 ml), the combined organic layer was washed with water (15 ml) followed by brine (15 ml) and concentrated under reduced pressure to give crude product, which was further purified by column chromatography on silica gel using hexane/EtOAc (5:1) as eluent to afford

title compound as yellow solid (1.02 g, 72%). mp 183-184°C; <sup>1</sup>H NMR (400 MHz,

CDCl<sub>3</sub>):  $\delta$  3.94 (s, 6H), 7.07 (dd, J = 2.0, 6.8 Hz, 4H), 8.14 (dd, J = 2.0, 6.8 Hz, 4H); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  55.6, 114.8, 121.3, 130.4, 163.5, 184.4, 196.3 ; Anal. Calcd. for C<sub>18</sub>H<sub>14</sub>O<sub>4</sub>: C, 73.46; H, 4.79. Found: C, 73.69; H, 4.81.

4.1.2. 3-(2,4-dimethoxyphenyl)-4-(4'-methoxyphenyl)cyclobut-3-ene-1,2-dione (4b)

A mixture of squaric acid (0.55 g, 4.8 mmol), thionyl dichloride (0.88 ml, 9.7 mmol) and DMF (5 drops) was heated at reflux for 3 h. The resultant mixture was cooled to 0°C. Anhydrous methylene chloride (6 ml) and anhydrous AlCl<sub>3</sub> (0.32 g, 2.4 mmol) were added to the reaction mixture. Then anisole (0.13 g, 1.2 mmol) was added to the mixture dropwise. The mixture was stirred at 0°C for 1.5 h. The resultant mixture was poured onto crushed ice and extracted with  $CH_2Cl_2$  (2×15 ml), the combined organic layer was washed with water (15 ml) followed by brine (15 ml) and concentrated under reduced pressure to give crude product, which was further recrystalized from  $CH_2Cl_2$ / hexane to give 3-chloro-4-(4-methoxyphenyl)cyclobut -3-ene-1,2-dione (**3**, 0.54 g, 50%) and was used in the next step without further purification.

**3** (0.54 g, 2.4 mmol) was dissolved in anhydrous methylene chloride (6 ml). The resultant mixture was cooled to 0°C. Anhydrous AlCl<sub>3</sub> (0.32 g, 2.4 mmol) was added to the reaction mixture. Then 1,3-dimethoxybenzene (0.33 g, 2.4 mmol) was added (dropwise) to the mixture. The mixture was stirred at 0°C for 1.5 h. The resultant mixture was poured onto crushed ice and extracted with  $CH_2Cl_2$  (2×15 ml), the combined organic layer was washed with water (15 ml) followed by brine (15 ml) and concentrated under reduced pressure to give crude product, which was further purified by column chromatography on silica gel using hexane/EtOAc (5:1) as eluent to afford

title compound as yellow solid (0.54 g, 69%). mp 182-183°C; <sup>1</sup>H NMR (400 MHz,

CDCl<sub>3</sub>):  $\delta$  3.69 (s, 3H), 3.90 (s, 3H), 3.92 (s, 3H), 6.55 (d, J = 2.4 Hz, 1H), 6.68 (dd, J = 2.4, 8.4 Hz, 1H), 6.97 (dd, J = 2.0, 6.8 Hz, 2H), 7.85 (dd, J = 2.0, 6.8 Hz, 2H), 7.97 (d, J = 8.4 Hz, 1H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  55.0, 55.5, 55.8, 98.7, 105.6, 111.3, 113.7, 122.6, 131.5, 131.8, 158.4, 163.2, 165.0, 182.2, 185.2, 196.5, 196.9;

Anal. Calcd. for C<sub>19</sub>H<sub>16</sub>O<sub>5</sub>: C, 70.36; H, 4.97. Found: C, 70.58; H, 5.01.

4.1.3. 3-(3,4-dimethoxyphenyl)-4-(4'-methoxyphenyl)cyclobut-3-ene-1,2-dione (4c)

The title compound was obtained as a yellow solid in 72% yield from 1,2-dimethoxybenzene using a procedure similar to that for compound **4b**. mp

165-166°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 3.92 (s, 3H), 3.94 (s, 3H), 4.00 (s, 3H),

7.02 (d, J = 8.4 Hz, 1H), 7.05 (dd, J = 2.0, 7.2 Hz, 2H), 7.63 (d, J = 2.0 Hz, 1H), 7.81 (dd, J = 2.0, 8.4 Hz, 1H), 8.14 (dd, J = 2.0, 7.2 Hz, 2H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  55.6, 56.1, 56.2, 110.5, 111.3, 114.7, 121.2, 121.3, 122.9, 130.5, 149.2, 153.2, 163.6, 184.4, 196.2, 196.2; Anal. Calcd. for C<sub>19</sub>H<sub>16</sub>O<sub>5</sub>: C, 70.36; H, 4.97. Found: C, 70.19; H, 4.96.

4.1.4. 3-(4'-methoxyphenyl)-4-(2,4,6-trimethoxyphenyl)cyclobut-3-ene-1,2-dione (4d)

The title compound was obtained as a yellow solid in 80% yield from 1,3,5-trimethoxybenzene using a procedure similar to that for compound **4b**. mp

185-187°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 3.77 (s, 6H), 3.90 (s, 3H), 3.92 (s, 3H),

6.23 (s, 2H), 6.96 (dd, J = 2.0, 6.8 Hz, 2H), 7.92 (dd, J = 2.0, 6.8 Hz, 2H); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  55.5, 55.6, 55.8, 90.8, 100.7, 113.9, 122.6, 131.5, 158.8, 163.3, 165.1, 184.6, 186.9, 195.9, 198.0; Anal. Calcd. for C<sub>20</sub>H<sub>18</sub>O<sub>6</sub>: C, 67.79; H, 5.12. Found: C, 67.74; H, 5.14.

4.1.5. 3-(4'-methoxyphenyl)-4-(2,3,4-trimethoxyphenyl)cyclobut-3-ene-1,2-dione (4e)

The title compound was obtained as a yellow solid in 62% yield from 1,2,3-trimethoxybenzene using a procedure similar to that for compound **4b**. mp

115-116°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 3.78 (s, 3H), 3.92 (s, 3H), 3.94 (s, 3H),

3.99 (s, 3H), 6.84 (d, J = 8.8 Hz, 1H), 6.99 (dd, J = 2.0, 6.8 Hz, 2H), 7.58 (d, J = 8.8 Hz, 1H), 8.01 (dd, J = 2.0, 6.8 Hz, 2H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  55.5, 56.3, 61.2, 61.4, 107.3, 114.1, 115.7, 121.8, 124.6, 131.6, 142.2, 151.8, 157.5, 163.7, 183.6, 186.3, 195.9, 197.2; Anal. Calcd. for C<sub>20</sub>H<sub>18</sub>O<sub>6</sub>: C, 67.79; H, 5.12. Found: C, 67.40; H, 5.16.

4.1.6. 3-phenyl-4-(3,4,5-trimethoxyphenyl)cyclobut-3-ene-1,2-dione (4f)

A mixture of squaric acid (0.55 g, 4.8 mmol), thionyl dichloride (0.88 ml, 9.7 mmol) and DMF (5 drops) was refluxed for 3 h. The resultant mixture was cooled to

0°C. Anhydrous methylene chloride (6 ml) and anhydrous AlCl<sub>3</sub> (0.32 g, 2.4 mmol)

were added to the reaction mixture. Then benzene (0.09g, 1.2mmol) was added to the mixture dropwise. The mixture was stirred at room temperature for 1.5 h. The resultant mixture was poured onto crushed ice and extracted with  $CH_2Cl_2$  (2×15 ml), the combined organic layer was washed with water (15 ml) followed by brine (15 ml) and concentrated under reduced pressure to give crude product, which was further recrystalized from  $CH_2Cl_2$ / hexane to give 3-chloro-4-(4-phenyl)cyclobut-3-ene-1,2-dione (0.51 g, 55%) and was used in the next step without further purification.

3-Chloro-4-(4-phenyl)cyclobut-3-ene-1,2-dione(0.46 g, 2.4 mmol) and

(3,4,5-trimethoxyphenyl)tri-*n*-butylstannane (1.43 g, 3.1 mmol) were dissolved in unhydrous CH<sub>3</sub>CN (6 ml) under N<sub>2</sub>. Then, the mixture was purged with N<sub>2</sub> for 5 min whereupon (CH<sub>3</sub>CN)<sub>2</sub>PdCl<sub>2</sub> (0.03 g, 0.12 mmol) was added and the reaction mixture was stirred at room temperature for 3 h. The mixture was filtered and purified by column chromatography on silica gel using hexane/EtOAc (5:1) as eluent to afford

title compound as yellow solid (0.51 g, 66%). mp 161-162°C; <sup>1</sup>H NMR (400 MHz,

CDCl<sub>3</sub>):  $\delta$  3.90 (s, 6H), 4.00 (s, 3H), 7.42 (s, 2H), 7.60 (m, 3H), 8.12 (m, 2H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  56.4, 61.0, 106.3, 123.0, 128.1, 128.5, 129.1, 132.9, 143.3, 153.7, 186.3, 186.7, 195.5, 196.2; Anal. Calcd. for C<sub>19</sub>H<sub>16</sub>O<sub>5</sub>: C, 70.36; H, 4.97. Found: C, 70.43; H, 4.88.

4.1.7. 3-(4'-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)cyclobut-3-ene-1,2-dione (**4g**) The title compound was obtained as a yellow solid in 35% yield from anisole

using a procedure similar to that for compound 4f. mp 154-155°C; <sup>1</sup>H NMR (400

MHz, CDCl<sub>3</sub>):  $\delta$  3.91 (s, 6H), 3.95 (s, 3H), 3.99 (s, 3H), 7.08 (dd, J = 2.0, 6.8 Hz, 2H), 7.37 (s, 2H), 8.20 (dd, J = 2.0, 6.8 Hz, 2H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  55.7, 56.4, 61.1, 105.6, 114.7, 121.0, 123.5, 130.7, 142.2, 153.6, 163.8, 184.6, 185.4, 195.8, 196.4; Anal. Calcd. for C<sub>20</sub>H<sub>18</sub>O<sub>6</sub>: C, 67.79; H, 5.12. Found: C, 67.71; H, 5.18.

4.1. 8. 3-(3,'4'-dimethoxyphenyl)-4-(3,4,5-trimethoxyphenyl)cyclobut-3-ene-1,2-dione (4h)

The title compound was obtained as a yellow solid in 38% yield from 1,2-dimethoxybenzene using a procedure similar to that for compound **4f**. mp

186-187°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 3.91 (s, 6H), 3.94 (s, 3H), 3.99 (s, 3H),

4.02 (s, 3H), 7.03 (d, J = 8.4 Hz, 1H), 7.38 (s, 2H), 7.69 (d, J = 2.0 Hz, 1H), 7.88 (dd, J = 2.0, 8.4 Hz, 1H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  56.2, 56.3, 56.4, 61.1, 105.6, 110.8, 111.2, 121.1, 123.1, 123.5, 142.3, 149.3, 153.5, 153.6, 184.7, 185.4, 195.8, 196.3; Anal. Calcd. for C<sub>21</sub>H<sub>20</sub>O<sub>7</sub>+0.9H<sub>2</sub>O: C, 62.96; H, 5.49. Found: C, 63.00; H, 5.18.

*4.1.9. 3-(2',4'-dimethoxyphenyl)-4-(3,4,5-trimethoxyphenyl)cyclobut-3-ene-1,2-dione (4i)* 

The title compound was obtained as a yellow solid in 42% yield from 1,3-dimethoxybenzene using a procedure similar to that for compound **4f**. mp

152-153°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 3.71(s, 3H), 3.85 (s, 6H), 3.93 (s, 3H),

3.96 (s, 3H), 6.56 (d, J = 2.0 Hz, 1H), 6.69 (dd, J = 2.0, 8.8 Hz, 1H), 7.16 (s, 2H) , 7.97 (d, J = 8.8 Hz, 1H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  55.2, 55.8, 56.3, 61.1, 98.6, 105.6, 106.9, 111.0, 124.7, 131.6, 142.0, 152.8, 158.6, 165.3, 183.7, 185.4, 196.4, 196.6; Anal. Calcd. for C<sub>21</sub>H<sub>20</sub>O<sub>7</sub>: C, 65.62; H, 5.24. Found: C, 65.65; H, 5.28.

4.1.10. 3-(3'-chloro-4'-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)cyclobut-3-ene-1,2 -dione (**4j**)

The title compound was obtained as a yellow solid in 46% yield from 1-chloro-2-methoxybenzene using a procedure similar to that for compound **4f**. mp

212-214°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 3.93(s, 6H), 4.01 (s, 3H), 4.05 (s, 3H),

7.12 (d, J = 8.4 Hz, 1H), 7.38 (s, 2H), 8.20 (dd, J = 2.0, 8.4 Hz, 1H), 8.24 (d, J = 2.0 Hz, 1H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  56.4, 56.5, 61.2, 105.7, 112.2, 121.6, 123.1, 123.7, 129.3, 129.8, 142.6, 153.6, 158.9, 183.8, 185.2, 195.7, 195.8; Anal. Calcd. for C<sub>20</sub>H<sub>17</sub>ClO<sub>6</sub>: C, 61.78; H, 4.41. Found: C, 62.07; H, 4.35.

*4.1.11. 3-(3'-fluoro-4'-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)cyclobut-3-ene-1,2 -dione* (*4k*)

The title compound was obtained as a yellow solid in 45% yield from 1-fluoro-2-methoxybenzene using a procedure similar to that for compound **4f**. mp

179-180°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 3.92(s, 6H), 4.00 (s, 3H), 4.04 (s, 3H),

7.14 (t, J = 8.4 Hz, 1H), 7.36 (s, 2H), 7.94 (dd, J = 2.0, 8.4 Hz, 1H), 8.07 (dt, J = 2.0, 8.4 Hz, 1H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  56.3, 56.4, 61.2, 105.7, 113.3, 113.4, 115.4, 115.6, 120.9, 121.0, 123.1, 126.2, 126.3, 142.6, 150.9, 152.0, 152.1, 153.4, 153.6, 184.1, 185.2, 195.7, 195.8; Anal. Calcd. for C<sub>20</sub>H<sub>17</sub>FO<sub>6</sub>: C, 64.51; H, 4.60. Found: C, 64.46; H, 4.61.

*4.1.12. 3-(4'-methoxyphenylthio)-4-(3,4,5-trimethoxyphenyl)cyclobut-3-ene-1,2dione* (**6**)

A mixture of squaric acid (5.7 g, 50 mmol), thionyl dichloride (9.0 ml, 99 mmol) and DMF (15 drops) was heated at reflux for 3 h. The mixture was cooled to room temperature, hexane (50 ml) was added to the mixture, filtered, concentration and after cooling was filtered under nitrogen, to give yellow crystalls of 3,4-dichlorocyclobut-3-ene-1,2-dione (2, 4.38 g, 58%) which was used in the next step without further purification.

2 (2.0g, 13.2 mmol) was dissolved in anhydrous THF (100 ml) under N<sub>2</sub> and the temperature was lowered to 0°C. *p*-Methoxybenzenethiol (0.9 ml, 6.6 mmol) and triethylamine (0.8 ml, 6.6 mmol) were sequentially added. The mixture was stirred at 0°C for 5 h whereupon the solvent was removed under reduced pressure. The remaining solid was extracted with EtOAc (2×150 ml), the organic extracts was passed through a aid pad and then the solvent was removed under reduced pressure, and further recrystalized from CH<sub>2</sub>Cl<sub>2</sub>/ hexane to give a pale yellow solid 3-chloro-4-(4-methoxyphenylthio)cyclobut-3-ene-1,2-dione (**5**, 2.86 g, 85%) which was used in the next step without further purification.

**5** (2.55 g, 10 mmol) and (3,4,5-trimethoxyphenyl)tri-*n*-butylstannane (5.94 g, 13 mmol) were dissolved in anhydrous CH<sub>3</sub>CN (30 ml) under N<sub>2</sub>. Then, the mixture was purged with N<sub>2</sub> for 5 min whereupon (CH<sub>3</sub>CN)<sub>2</sub>PdCl<sub>2</sub> (0.13 g, 0.5 mmol) was added and the reaction mixture was stirred at room temperature for 3 h. The mixture then was filtered and purified by column chromatography on silica gel using hexane/EtOAc (5:1) as eluent to afford the title compound as a yellow solid (3.09 g,

80%). mp 184-185°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 3.89 (s, 3H), 3.98 (s, 6H), 3.99

(s, 3H), 6.99 (dd, J = 2.0, 6.8 Hz, 2H), 7.34 (s, 2H), 7.58 (dd, J = 2.0, 6.8 Hz, 2H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  55.4, 56.4, 61.2, 106.2, 115.0, 115.2, 123.7, 135.5, 142.3, 153.6, 161.6, 180.6, 188.7, 190.8, 192.3; Anal. Calcd. for C<sub>20</sub>H<sub>18</sub>O<sub>6</sub>S: C, 62.16; H, 4.70. Found: C, 61.86; H, 4.59.

4.1.13. 3,4-bis(3,4,5-trimethoxyphenyl)cyclobut-3-ene-1,2-dione (4l)

**6** (0.39 g, 1 mmol) and 3,4,5-trimethoxyphenylboronic acid (0.53 g, 2.5 mmol) were dissolved in dry THF (6 ml) under  $N_2$ . Then, the mixture was purged with  $N_2$  for 10 min whereupon, Pd<sub>2</sub>bda<sub>3</sub> (9.16 mg, 1%), TFP (6.96 mg, 3%) and CuTC (0.57g, 3

mmol) were added. The reaction mixture was heated at 50°C for 20 h, cooled to room

temperature, and then quenched with an aqueous  $NH_4Cl$  solution. Then, it was extracted with EtOAc (2×30 ml), dried over MgSO<sub>4</sub>, filtered and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel using hexane/EtOAc (3:1) as eluent to afford title

compound as yellow solid (0.29 g, 70%). mp 161-163°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):

δ 3.92 (s, 12H), 4.00 (s, 6H), 7.41 (s, 4H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>): δ 56.5, 61.2, 105.9, 123.3, 142.7, 153.6, 185.6, 195.9; Anal. Calcd. for C<sub>22</sub>H<sub>22</sub>O<sub>8</sub>: C, 63.76; H, 5.35. Found: C, 63.67; H, 5.31.

4.1.14. 5-(3,4-dioxo-2-(3,4,5-trimethoxyphenyl)cyclobut-1-enyl)-2-methoxyphenyl acetate (**4m**)

The title compound was obtained as a yellow solid in 63% yield from 3-acetoxy-4-methoxyphenylboronic acid using a procedure similar to that of

compound **4I**. mp 159-160°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 2.36 (s, 3H), 3.91 (s,

6H), 3.97 (s, 3H), 4.00 (s, 3H), 7.14 (d, J = 8.8 Hz, 1H), 7.35 (s, 2H), 7.89 (d, J = 2.4 Hz, 1H), 8.17 (dd, J = 2.4, 8.8 Hz, 1H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  20.6, 56.3, 56.4, 61.2, 105.5, 112.6, 121.0, 122.9, 123.2, 128.6, 104.1, 142.4, 153.6, 155.4, 168.7, 184.4, 185.1, 195.7, 195.9; Anal. Calcd. for C<sub>22</sub>H<sub>20</sub>O<sub>8</sub>: C, 64.07; H, 4.89. Found: C, 64.17; H, 4.83.

4.1.15. 3-(3'-hydroxy-4'-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)cyclobut-3ene-1,2-dione (**4n**)

A solution of 4m (0.5 g, 1.2 mmol), 10% NaHCO<sub>3</sub> (3 ml, 1.8 mmol), and MeOH (2 ml) was allowed to reflux for 1.5 h. Cooled to room temperature and filtered and washed with water to give title compound as yellow solid (0.38 g, 85%). mp

223-224°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 3.92 (s, 6H), 4.00 (s, 3H), 4.04 (s, 3H),

5.76 (s, 1H), 7.03 (d, J = 8.4 Hz, 1H), 7.40 (s, 2H), 7.73 (d, J = 2.0 Hz, 1H), 7.88 (dd, J = 2.0, 8.4 Hz, 1H); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  56.3, 56.4, 60.8, 105.7, 112.8, 114.5, 121.0, 122.1, 123.7, 141.7, 147.5, 153.1, 153.6, 183.9, 185.3, 196.0, 196.9; Anal. Calcd. for C<sub>20</sub>H<sub>18</sub>O<sub>7</sub>: C, 64.86; H, 4.90. Found: C, 64.95; H, 4.85.

4.1.16. 3-(4'-methoxy-3'-nitrophenyl)-4-(3,4,5-trimethoxyphenyl)cyclobut-3-ene-1,2-dione (**40**)

The title compound was obtained as a yellow solid in 65% yield from

4-methoxy-3-nitrophenylboronic acid using a procedure similar to that of compound

**4I**. mp 233-234°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 3.95 (s, 6H), 4.02 (s, 3H), 4.12 (s,

3H), 7.32 (d, J = 8.8 Hz, 1H), 7.38 (s, 2H), 8.51 (dd, J = 2.0, 8.8 Hz, 1H), 8.67 (d, J = 2.0 Hz, 1H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  56.7, 57.9, 60.9, 106.5, 116.1, 120.6, 123.2, 125.0, 134.6, 139.9, 142.9, 153.8, 155.8, 183.1, 185.6, 195.7, 196.0; Anal. Calcd. for C<sub>20</sub>H<sub>17</sub>NO<sub>8</sub>: C, 60.15; H, 4.29; N, 3.51. Found: C, 60.09; H, 4.24; N, 3.69. *4.1.17. 3-(3'-amino-4'-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)cyclobut-3-ene-1,2-dione* (**4***p*)

A solution of 40 (0.2 g, 0.5 mmol) in ethyl acetate (30 mL) and EtOH (20 ml) was hydrogenated (40 psi) at room temperature over 10% Pd/C (0.02 mg) for 1.5 h. The black suspension was filtered through a filter aid pad and the filtrate was concentrated under reduced pressure to give the amino compound as yellow solid (0.15 g, 83%). mp

198-200°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 3.92 (s, 6H), 3.99 (s, 6H), 4.02 (s, 2H),

6.93 (d, J = 8.4 Hz, 1H), 7.41 (s, 2H), 7.54 (d, J = 2.0 Hz, 1H), 7.67 (dd, J = 2.0, 8.4 Hz, 1H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  56.1, 56.4, 60.8, 105.7, 111.0, 112.0, 119.1, 121.2, 123.8, 139.0, 141.7, 151.4, 153.5, 183.5, 186.0, 196.2, 197.1; Anal. Calcd. for C<sub>20</sub>H<sub>19</sub>NO<sub>6</sub> + 0.8 H<sub>2</sub>O: C, 63.73; H, 5.25; N, 3.54. Found: C, 63.61; H, 5.14; N, 3.70. **4.1.18.** *3*-(1*H*-indol-5'-yl)-4-(3,4,5-trimethoxyphenyl)cyclobut-3-ene-1,2-dione (**4***q*)

The title compound was obtained as a yellow solid in 66% yield from 1H-indol-5-ylboronic acid using a procedure similar to that of compound **4**l. mp

228-230°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  3.91 (s, 6H), 4.00 (s, 3H), 6.73 (t, J = 2.4

Hz, 1H), 7.36 (t, J = 2.4 Hz, 1H), 7.47 (s, 2H), 7.56 (d, J = 8.4 Hz, 1H), 8.02 (dd, J = 1.6, 8.4 Hz, 1H), 8.51 (s, 1H), 8.66 (s, 1H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  56.4, 60.8, 103.7, 105.6, 113.0, 119.5, 121.6, 122.8, 124.0, 128.3, 139.2, 141.7, 153.6, 183.5, 187.2, 196.0, 197.4; Anal. Calcd. for C<sub>21</sub>H<sub>17</sub>NO<sub>5</sub> + 0.3 H<sub>2</sub>O: C, 68.40; H, 4.81; N, 3.80. Found: C, 68.26; H, 4.70; N, 3.73.

4.1.19. 3-(1H-indol-6'-yl)-4-(3,4,5-trimethoxyphenyl)cyclobut-3-ene-1,2-dione (4r)

The title compound was obtained as a yellow solid in 53% yield from 1*H*-indol-6-ylboronic acid using a procedure similar to that of compound **4**l. mp

193-195°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  3.92 (s, 6H), 4.00 (s, 3H), 6.91 (t, J = 2.4

Hz, 1H), 7.47 (s, 2H), 7.51 (t, J = 2.4 Hz, 1H), 7.78 (d, J = 8.4 Hz, 1H), 7.87 (dd, J = 1.6, 8.4 Hz, 1H), 8.50 (s, 1H), 8.72 (s, 1H); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  56.5, 60.8, 102.9, 105.6, 113.2, 119.2, 120.8, 121.3, 124.0, 131.3, 132.4, 135.9, 141.6, 153.6, 183.8, 186.8, 195.9, 197.3; Anal. Calcd. for C<sub>21</sub>H<sub>17</sub>NO<sub>5</sub> + 0.6 H<sub>2</sub>O: C, 67.41; H, 4.90; N, 3.74. Found: C, 67.56; H, 4.77; N, 3.86.

4.2. Biology

#### 4.2.1. cell lines

Human tumor cell lines CEM (leukemia), HepG2(liver cancer), MCF-7 (breast cancer), SW-1990(pancreatic cancer) and HCT116 (colon adenocarcinoma) were from the American Type Culture Collection. The human hepatoma cells Bel-7402 and

SMMC-7721 were provided by the Cancer Institute of Chinese Academy of Medical Sciences (Beijing, China). The cell lines were cultured in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 100 units/mL penicillin, and 100 Ag/mL streptomycin. Cells (cultured in 5% CO<sub>2</sub> at 37°C) in exponential growth were used in all experiments.

## 4.2.2. Cytotoxicity assay

Cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously [29] . Briefly, cells were seeded into the 96-well culture plates at 10000 per well followed by the treatment of test compounds at 11 increasing concentrations between 0-10  $\mu$ M at 37 °C. After 72 h, 20  $\mu$ L of MTT PBS solution (5 mg/mL) was added into each well. After additional 4 h incubation at 37 °C, the supernatants were removed gently and 150  $\mu$ L of DMSO was added into each well. The plate was then incubated at room temperature for 10 min with 300 rpm shaking and instantly read with a microplate reader (Molecular Device, US) at wavelength of 590 nm with a reference filter of 620 nm. The IC<sub>50</sub> values were defined as 50% inhibition of cells growth compared with untreated controls, calculated by regression analysis, and were determined in duplicates.

## 4.2.3. Cell cycle analysis

Cell cycle analysis was conducted followed a method reported previously [29]. Briefly, MCF-7 cells were plated in 6-well plates in growth medium at a density of 500,000 cells per well overnight, followed by treatment with 0.08  $\mu$ M of compound **CA4** and 0.02  $\mu$ M of compound **4n**. After 24 h, cells were fixed in 70% ethanol overnight and then treated with 50  $\mu$ g/mL propidium iodide containing RNase A for 0.5 h at room temperature. Stained cell samples were immediately subjected to an analysis in a flow cytometer (Beckman Coulter). For each sample, 10,000 cells were (version 2.0).

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Some novel 3,4-diaryl squaric acid analogues were synthesized. ► Compounds were tested for cytotoxic activities against human tumour cell lines. ► Compounds 4g, 4k, 4m, 4n, 4p, 4q and 4r exhibit strong activities against human leukemia cells with IC50 values of ≤20 nM. ► Compounds 4k, 4n, 4p, 4q and 4r showed potent activities against a panel of human tumor cell lines.