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# Synthesis of Hirtellanine B, an Isoflavonoid with Potent Antiproliferative Effects in Cancer Cells

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We reported the first total synthesis of hirtellanine B, using oxidative coupling for the key reactions, which resulted in a high yield. The antiproliferative activity of hirtellanine B against Jurkat cells, Raji cells and K562 cells were also investigated. It was found that hirtellanine B could induce G2/M arrest and apoptosis in human lymphoid/leukemia tumor cells.

**Keywords** hirtellanine B, synthesis, antiproliferation, G2/M arrest, cancer cell

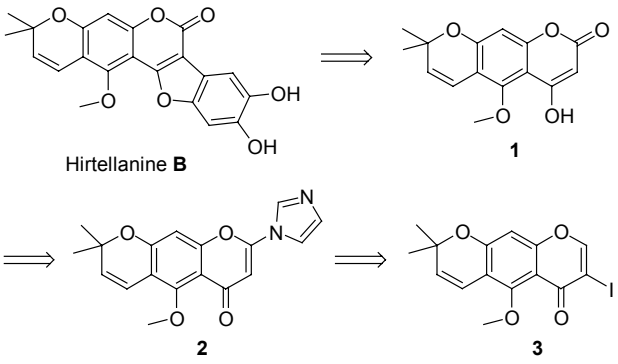
## Introduction

Hirtellanine B is an isoflavonoid isolated from the roots of *Campylotropis hirtella* (Franch.) Schindl. A previous study indicated that hirtellanine B showed moderate B lymphocyte suppression activity ($IC_{50}$: 3.00 μmol•$L^{-1}$) and T lymphocyte suppression activity ($IC_{50}$: 9.55 μmol•$L^{-1}$).[1] However, our *in vitro* studies unveiled that hirtellanine B inhibits the proliferation of the Jurkat, Raji and K562 cell lines. Furthermore, this compound was found to induce G2/M arrest in these cancer cells. Interestingly, all other flavanones isolated from this plant, including hirtellanine A[1-4] do not show any effects of inducing G2/M arrest of the above-mentioned tumor cells. Thus, it is of interest to further explore the synthesis and structure-function relationship of hirtellanine B. In this letter, we describe the first total synthesis of hirtellanine B and report its antiproliferative effects and induction of G2/M arrest in human lymphoid/leukemia tumor cells.

## Results and Discussion

Our retrosynthetic analysis of hirtellanine B is depicted in Scheme 1. The key intermediate 3-iodo-5-methoxy-dimethylchromene pyran-4-one **3** can be prepared from commercially available acetylphloroglucinol.[4] The compound **3** can readily be converted into intermediate **2** according to the literature,[5] and the compound **1** can be obtained from the hydrolysis of compound **2**. Finally, we anticipate the intermediate **1** can be coupled with *o*-dihydroxybenzene to form hirtellanine B.

**Scheme 1** Retrosynthetic analysis of hirtellanine B




3-Iodo-5-methoxy-dimethylchromene pyran-4-one **3** was obtained from acetylphloroglucinol in five steps with an overall yield of 71%.[4] As shown in Scheme 2, after treated with imidazole in dimethyl formamide, **3** was smoothly converted into **2** with a 95% yield.[6] The subsequent acid hydrolysis of **2** afforded coumarin intermediate **1** with a 93% yield. For the hydrolysis reaction, both reaction conditions of 10% aqueous HCl in EtOH and 10% HCl in THF were tried, however, large amounts of by-products were observed; when AcOH (acetic acid glacial) was used, only target compound was obtained. The final coupling of **1** and *o*-dihydroxybenzene was achieved in the presence of $K_3Fe(CN)_6$ to deliver hirtellanine B with a 69% yield.[6] In the oxidative coupling step, catechol was oxidized by potassium ferricyanide, and *o*-benzoquinone was generated *in-situ* which participated into Michael addition with 4-hydroxycoumarin **1** to form hirtellanine B. The spectroscopic data of synthesized hirtellanine B were iden-

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tical to those obtained from the authentic sample from plant material. Thus, we have developed the first synthetic hirtellanine B, using oxidative coupling as the key reaction. The eight-step synthetic process converted acetylphloroglucinol to hirtellanine B in an overall yield of 41%.

In our preliminary studies, hirtellanine B was shown to inhibit human lymphoid/leukemia tumor cell proliferation.[7] Significant growth inhibition was observed after 24 h of treatment with hirtellanine B in tumor cell lines. The concentration of hirtellanine B required for 50% inhibition of cell growth ($CC_{50}$) was (7.17±0.04) μmol•L$^{-1}$ in Jurkat cells, (6.58±0.40) μmol•L$^{-1}$ in Raji cells and (6.76±0.16) μmol•L$^{-1}$ in K562 cells.

To investigate the mechanism of hirtellanine B-mediated cell growth inhibition, we examined cell cycle alterations by flow cytometry.[8,9] The effects of hirtellanine B on cell cycle progression of Jurkat, Raji and K562 cells are shown in Figure 1 and Table 1. After 24 h of growth, the $G_2$/M phase cell population of control Jurkat cells was 13.50%. Meanwhile, the $G_2$/M phase cell population of hirtellanine B-treated cells increased in a dose-dependent manner. A concentration of 2.5 μmol•L$^{-1}$ hirtellanine B resulted in the highest percentage of cells (69.05%) in $G_2$/M phase of the cell cycle. As the $G_2$/M phase population increased, the $G_1$ phase cell population decreased, while the S phase cell population showed slight changes within 24 h.

We also investigated the inhibitory effects of hirtellanine B on Raji cells, as shown in Table 1 and Figure 1. After 24 h of incubation, the G2/M phase cell population of the control cells was 10.50%, while the G2/M phase cell population of 2.5 μmol•L$^{-1}$ hirtellanine B-treated cells increased to 31.93%. In addition, when the $G_2$/M phase population increased, the $G_1$ phase cell population decreased, and the S phase cell population remained almost unchanged.

Similar findings were obtained with K562 cells. Compared with control cells, after 24 h incubation, the $G_2$/M phase cell population of the 2.5 μmol•L$^{-1}$ hirtellanine B-treated K562 cells increased to 34.26%, whereas the cell population both at $G_1$ phase and S phase decreased.

This $G_2$/M cell cycle arrest was also detected after 48 h of treatment. At the highest concentration (2.5 μmol•L$^{-1}$) of hirtellanine B, the proportion of cells in $G_2$/M phase increased to 79.38%, compared with the

**Scheme 2** The synthesis of hirtellanine B

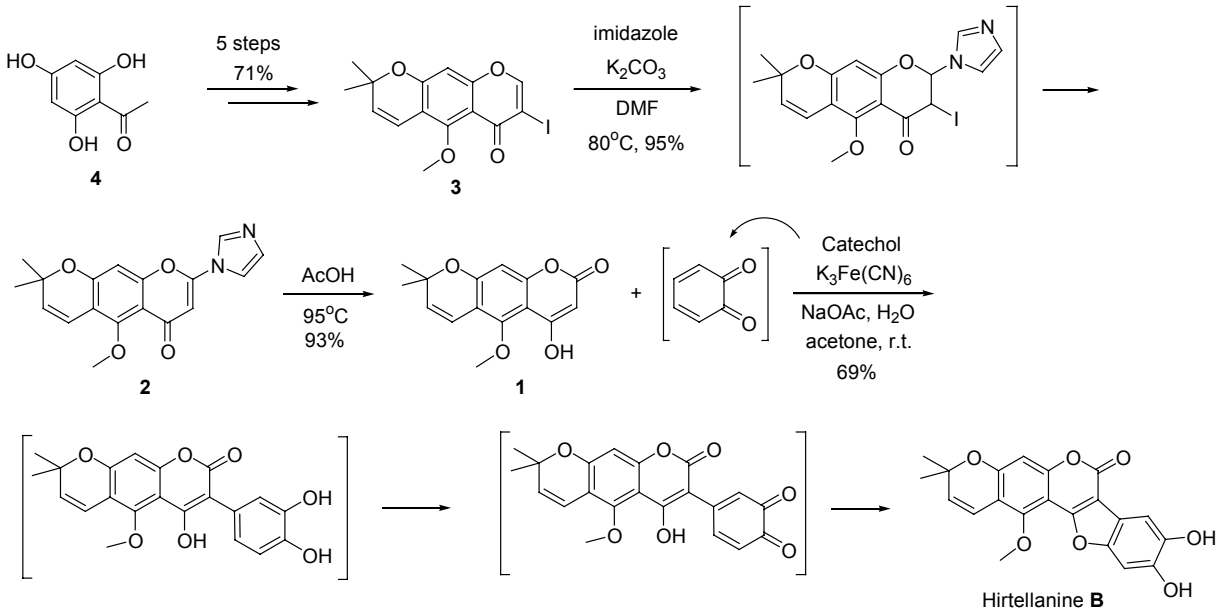


**Table 1** The effects of hirtellanine B on cell cycle progression[a]

| | Jurkat cell line/% | | | Raji cell line/% | | | K562 cell line/% | | |
|---|---|---|---|---|---|---|---|---|---|
| | $G_0/G_1$ | *S*/% | $G_2/M$ | $G_0/G_1$ | *S*/% | $G_2$/M | $G_0/G_1$ | *S*/% | $G_2$/M |
| Control | 57.63 | 28.87 | 13.50 | 56.63 | 32.87 | 10.50 | 42.51 | 42.59 | 14.90 |
| Hirtellanine B, 2.5 μmol•L$^{-1}$ | 12.75 | 18.20 | 69.05 | 41.98 | 26.08 | 31.93 | 32.25 | 33.49 | 34.26 |
| Hirtellanine B, 1.25 μmol•L$^{-1}$ | 40.55 | 11.28 | 48.16 | 57.30 | 23.11 | 19.59 | 40.21 | 41.73 | 18.06 |
| Hirtellanine B, 0.63 μmol•L$^{-1}$ | 58.18 | 27.17 | 14.65 | 69.29 | 27.03 | 12.67 | 43.12 | 41.32 | 15.56 |

[a] Cells were harvested after treated with the indicated concentrations of hirtellanine B. Analysis of the cell cycle distribution was performed using an equal number of cells ($1\times10^4$) by flow cytometry, after staining the DNA with propidium iodide.

  

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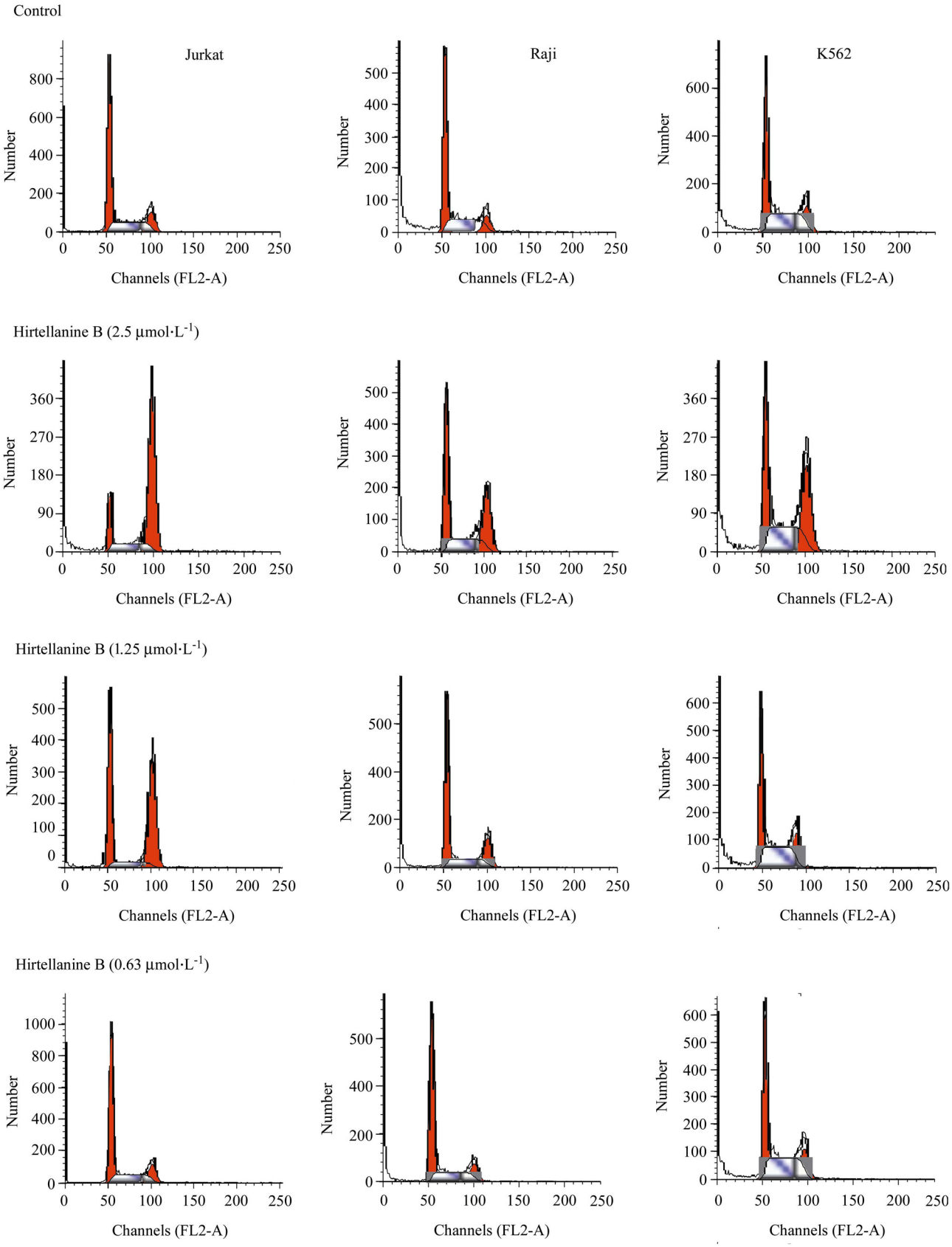


**Figure 1** Flow cytometric analysis of the cell cycle distribution in Jurkat cells, Raji cells and K562 cells after treatment with various concentrations of hirtellanine **B**.

control cells (8.93%). These results indicated that the cytotoxicity of hirtellanine B towards human lymphoid/leukemia tumor cells is mainly attributable to the induction in $G_2$/M arrest.

  

## Methods

### Cell lines and culture

The human lymphoblast-like T-cell line Jurkat, the human lymphoma B-cell line Raji and the human erythroleukemia cell line K562 were obtained from ATCC (Manassas, VA, USA) and were cultured in RPMI 1640 medium (Gibco Invitrogen Co., Grand Island, NY, USA) supplemented with 10% fetal calf serum (Hyclone, UT, USA), 100 U•mL$^{-1}$ of penicillin and 100 μg•mL$^{-1}$ of streptomycin in a humidified atmosphere at 37 ℃ in 5% $CO_2$.

### MTT proliferation assay

To measure the cytotoxicity of a compound, Jurkat, Raji and K562 cells were seeded ($2\times10^4$ cells•mL$^{-1}$) in 96-well microtiter plates and incubated with different concentrations of the compound for 48 h. Then, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, USA) was added 4 h before the end of culture. After centrifugation, the supernatants were discarded and the cells were dissolved in dimethyl sulfoxide. Optical density values were read at 570 nm, and the percentage of cell death was calculated.

### Cell cycle analysis

To analyze the effect on the cell cycle of the compound, Jurkat, Raji and K562 cells were seeded ($2\times10^5$ cells•mL$^{-1}$) in 24-well plates and incubated with different concentrations of the compound for 24 h. Cells were then harvested, washed with cold phosphate-buffered saline and fixed in ice-cold 70% ethanol overnight at 4 ℃. Cells were stained with 50 μg•mL$^{-1}$ propidium iodide (PI) and 100 U•mL$^{-1}$ RNase, and analyzed (10000 events were collected per sample) in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using the CellQuest software.

## Conclusions

In summary, we report the first practical synthesis of hirtellanine B, involving an eight-step reaction and resulting in an overall yield of 41%. We have also demonstrated the inhibitory effect of hirtellanine B on Jurkat, Raji and K562 cell proliferation. Its cytotoxicity towards these tumor cells is mainly attributable to the induction of G2/M arrest.

## Experimental

### General methods

Reagents were purchased from commercial vendors and used as received unless otherwise stated. Acetone and DMF were dried by 4 Å molecular sieves. Reactions were monitored by analytical thin-layer chromatography (TLC) with silica gel GF254 with a layer thickness of 0.25 mm purchased from Tsingdao Haiyang Co. Ltd. All reactions were stirred with PTFE coated magnetic stir bars and Heidolph® MR3001® stirrers. Removal of solvents was typically accomplished using a rotary evaporator. Anhydrous $Na_2SO_4$ was used to dry all of the organic extracting layer for workup. All the concentration was performed under reduced pressure. NMR spectra were recorded at a Varian Mercury Plus 400 MHz instrument with TMS as an internal standard unless otherwise indicated. MS (ESI-MS) were recorded on a Varian 1200L Quadrupole or Agileant 1100 LC/MSD or Waters Acquity SQD MS spectrometer.

**2-Imidazol-5-methoxyl-8,8-dimethyl-8*H*-pyrano-[3,2-g]chromone (2)** A mixture of **3** (285 mg, 0.74 mmol), imidazole (249 mg, 1.48 mmol) and $K_2CO_3$ (1.02 g, 7.40 mmol) in DMF (12 mL) was stirred at 80 ℃ for 2 h. After cooling, the reaction solution was poured into ice water (30 mL) under stirring. After stirring for 5 min, the mixture was standed for 30 min. The filtration gave the silver-gray solid product (215 mg), and the filtrate was extract with ethyl acetate and gave the white solid (15 mg) after concentration and purification with prep. TLC with ethyl acetate and petroleum ether (1∶3). The overall yield was 95.8%. m.p. 191—192 ℃; $^1$H NMR (400 MHz, Acetone-$d_6$) $\delta$: 8.42 (s, 1H), 7.82 (s, 1H), 7.19 (s, 1H), 6.83 (s, 1H), 6.74 (d, $J$=10.0 Hz, 1H), 6.41 (s, 1H), 5.92 (d, $J$=10.4 Hz, 1H), 3.86 (s, 3H), 1.47 (s, 6H); $^{13}$C NMR (100 MHz, acetone-$d_6$) $\delta$: 176.11, 171.94, 158.76, 157.37, 155.93, 152.62, 135.90, 131.96, 131.68, 116.93, 116.12, 114.28, 101.36, 97.78, 78.52, 62.77, 28.41; IR (film) $\nu_{max}$: 3454, 3119, 3065, 2980, 1655, 1597, 1464, 1412, 1288, 1120, 864, 646 cm$^{-1}$; HRMS (ESI$^+$) calcd for $C_{18}H_{17}N_2O_4$ ([M+1]$^+$) 325.1183, found 325.1187.

**4-Hydroxycoumarin (1)** The solution of **2** (80 mg, 0.25 mmol) in AcOH (acetic acid glacial) (5 mL) was heated at 95 ℃ for 30 h. The reaction solution was cooled to r.t., the ice water (15 mL) was added under stirring and large amount of solid was formed. Further stirring for 20 min and then standing for 30 min, the solid was filtrated and the solid cake was washed with cold water to give the off-white solid product (55 mg). The filtrate was treated with ethyl acetate to afford additional white solid (8 mg) after purification by perp. TLC with ethyl acetate and petroleum ether (1∶2). The overall yield was 93.1%. m.p. 124—125 ℃; $^1$H NMR (400 MHz, Acetone-$d_6$) $\delta$: 6.66 (d, $J$=10.0 Hz, 1H), 6.51 (s, 1H), 5.92 (d, $J$=10.0 Hz, 1H), 5.41 (s, 1H), 3.98 (s, 3H), 1.47 (s, 6H); $^{13}$C NMR (100 MHz, acetone-$d_6$) $\delta$: 166.13, 161.51, 157.77, 155.94, 153.51, 131.21, 115.52, 111.49, 102.44, 100.87, 90.11, 77.74, 63.94, 27.35; IR (film) $\nu_{max}$: 3524, 3346, 2978, 2854, 2613, 1678, 1616, 1319, 1165, 1083, 939, 825; ESI-MS $m/z$: 275.3 ([M+1]$^+$, 100%). Spectral data were identical to the literature data.[10]

**Hirtellanine B** Compound **1** (50 mg, 0.18 mmol) and *o*-diphenol (35 mg, 0.32 mmol) were dissolved into acetone (7 mL). The solution of NaOAc (177 mg, 2.15

  

mmol) in $H_2O$ (2 mL) was added, and subsequently, the solution of $K_3Fe(CN)_6$ (214 mg, 0.65 mmol) in 10 mL $H_2O$ was added dropwise in 30 min. After stirring for 2 h, the mixture was filtered and washed with acetone. The filtrate was concentrated under reduced pressure. Ethyl acetate (50 mL) was added, and washed with water and brine, respectively. After dryness and concentration, the residue was suspended into DCM (6 mL). After filtration, the light yellow solid (48 mg, 69%) was obtained. m.p. 283—285 ℃; $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$: 9.30—9.70 (br, s, 2H), 7.24 (s, 1H), 7.17 (s, 1H), 6.77 (s, 1H), 6.67 (d, $J$=10.4 Hz, 1H), 5.94 (d, $J$=10.0 Hz, 1H), 3.92 (s, 3H), 1.43 (s, 6H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$: 158.03, 157.84, 156.50, 154.44, 151.29, 149.89, 146.64, 145.23, 132.23, 115.65, 114.23, 112.76, 105.29, 103.97, 102.29, 101.51, 99.52, 78.39, 63.80, 28.45; IR (film) $\nu_{max}$: 3502, 3414, 3126, 1707, 1624, 1371, 1280, 1122, 862; HRMS (ESI$^+$) calcd for $C_{21}H_{17}O_7$ ($[M+1]^+$) 381.0969, found 381.0976.

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