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## Four new isocoumarins from *Cajanus cajan*†

Liyuan Yao,<sup>abc</sup> Aikebaier Jumai,<sup>ab</sup> Xiaobao Huang,<sup>ae</sup> Tingting Lin,<sup>a</sup> Zhengguo Tao<sup>d</sup> and Sheng-Xiang Qiu<sup>id</sup>\*<sup>ab</sup>

Four novel new isocoumarins, cajanolactone B, C, D1 and D2 (**1–4**), were isolated from ethanolic extracts of the leaves of *Cajanus cajan*. The structural elucidation has been completed mainly depending on extensive spectroscopic analysis including UV, IR, NMR (1D and 2D), HRESIMS and chiral analysis. Notably, all these new isocoumarins were found to exist in racemic forms, among which compounds **3** and **4** share the same planar structure. This finding suggests that at least the biosynthesis of isocoumarin in *C. cajan* is chiral tolerant. A plausible biogenetic pathway of compounds **1–4** is proposed.

## Introduction

Isocoumarins, as isomers of coumarins, feature an inverted lactone ring, namely 1*H*-2-benzo[*c*]pyran-1-one, which is a privileged scaffold offering a prodigious diversity of chemical structures that encompass a wide spectrum of pharmacological activities, such as antitumor, anticancer, antiviral, anti-inflammatory and antidiabetic *etc.*<sup>1–5</sup>

Pigeon pea [*Cajanus cajan* (L.) Millsp.], belonging to the Leguminosae family, is one of the most valuable legume food crops in the tropical world and a resource for traditional Chinese medicine. Its extracts and active ingredients have received increasing attention in recent years due to their various biological activities,<sup>6</sup> among which the main components such as cajanin stilbene acid, cajanonic acid A, longistylin A and pinostrobin have exhibited excellent bioactivities including antioxidant,<sup>7</sup> anti-cancer,<sup>8</sup> bactericidal,<sup>9</sup> antiviral,<sup>10</sup> lipid-lowering,<sup>11</sup> neuroprotective<sup>12</sup> and so on.

Previous studies on the phytochemistry of *C. cajan* from our and other research groups have focused on its main components, namely, flavonoids and stilbenes. In contrast, there has been limited exploration of smaller-proportion constituents such as isocoumarins. To the best of our knowledge, the only isocoumarin isolated from *C. cajan* was named cajanolactone A (CLA), which was found to promote osteoblast differentiation in hBMSCs through activating Wnt/LRP5/β-catenin signaling

transduction.<sup>13</sup> Our ongoing search for more structurally diverse compounds from *C. cajan* resulted in the isolation of four new isocoumarins (**1–4**) (Fig. 1), the isolation, structural elucidation of which are reported in the present report.

## Results and discussion

### Structure elucidation and identification

Compound **1** was purified as a colorless powder. Its molecular formula was determined to be C<sub>21</sub>H<sub>20</sub>O<sub>5</sub> by HRESIMS from the ion peak at *m/z* 353.1382 [M + H]<sup>+</sup> (calcd 353.1384) with twelve degrees of unsaturation. The typical IR resonance absorptions at 3374 and 1668 cm<sup>−1</sup> suggested the existence of hydroxyl and carbonyl functionalities. Inspection of the 1D NMR data (Table 1) revealed a series of characteristic resonances as follow: a penta-substituted benzene ring [ $\delta_{\text{H}}$  6.56 (1H, s)], a mono-substituted benzene ring [ $\delta_{\text{H}}$  7.42–7.49 (3H, m),  $\delta_{\text{H}}$  7.84–7.86 (2H, m)], a methoxy ( $\delta_{\text{C}}$  56.2), as well as a chelated hydroxyl group [ $\delta_{\text{H}}$  11.42 (1H, s)]. Signals at 2.95 (1H, dd, *J* = 14.2, 8.5 Hz), 3.09 (1H, dd, *J* = 14.3, 4.2 Hz), 4.26 (1H, dd, *J* = 8.3, 4.0 Hz), 4.98 (1H, s), 4.87 (1H, s), and 3.92 (3H, s), coupling with carbon resonances at  $\delta_{\text{C}}$  31.6, 76.1, 147.6, 110.8, 18.4 ppm denoted the presence of an isopentenyl group. Furthermore, on the basis of twelve indices of hydrogen deficiency, in conjunction with the high value of the corresponding carbonyl resonance at  $\delta_{\text{C}}$  166.6 ppm, a lactone ring was proposed.

The identification of the planar structure of **1** was accomplished by a comprehensive interpretation of its 2D NMR spectra (Fig. 2). The HMBC contour maps allowed determination of the position of the following fragments: the conclusive correlations between H-7 to C-2, C-6 and C-8, H-10/14 to C-8, H<sub>2</sub>-1'to C-1, C-5 and C-6, together with 5-OCH<sub>3</sub> to C-5, strongly suggested that **1** has a structure very similar to that of CLA. The only difference between **1** and CLA is that **1** has an extra isopentenyl group that can rationally attributed at the C-6.

Additionally, the optical rotation of **1** was measured as being close to zero, suggesting it might be a pair of racemates with

<sup>a</sup>State Key Laboratory of Plant Diversity and Specialty Crops & Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, Chinese Academy of Sciences, Program for Natural Product Chemical Biology, Guangzhou 510650, China. E-mail: sxqiu@scbg.ac.cn; Fax: +86-20-37081190; Tel: +86-13924031914

<sup>b</sup>South China National Botanical Garden, Guangzhou 510650, P. R. China

<sup>c</sup>Graduate University of Chinese Academy of Sciences, Beijing 100049, P. R. China

<sup>d</sup>Guangzhou Leader Biotechnology Co., LTD, Guangzhou, 510650, China

<sup>e</sup>Visiting Student from the Department of Chemistry, University of Wisconsin–Madison, USA

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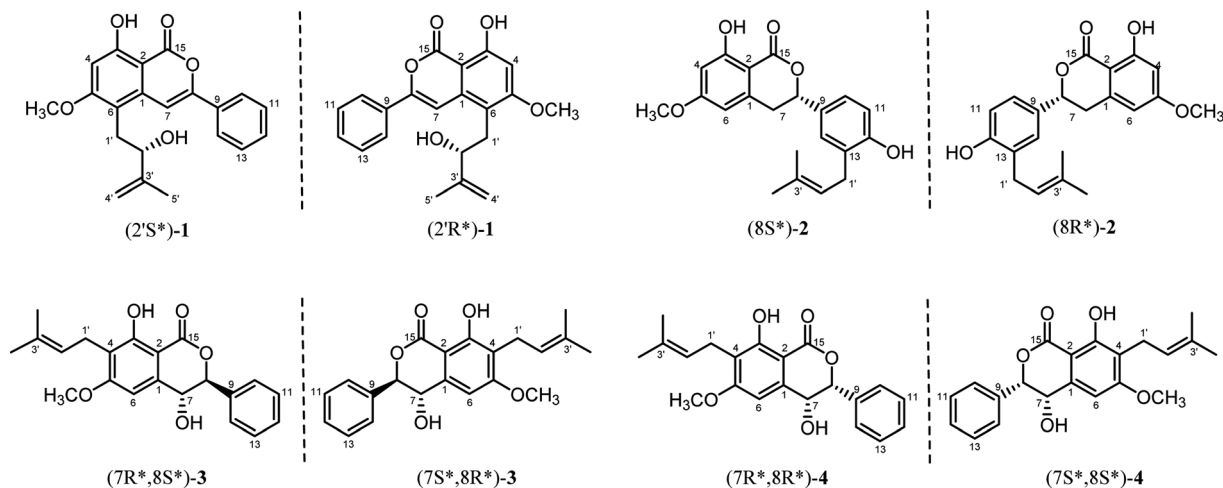


Fig. 1 Chemical structures (relative configurations) of compounds 1–4.

Table 1  $^1\text{H}$  (500 MHz) and  $^{13}\text{C}$  NMR (125 MHz) data of compounds 1 and 2,  $\delta$  in ppm in  $\text{CDCl}_3$

1			2		
No.	$\delta_{\text{H}}$ ( $J$ in Hz)	$\delta_{\text{C}}$ , type	No.	$\delta_{\text{H}}$ ( $J$ in Hz)	$\delta_{\text{C}}$ , type
1		137.4, C	1		141.2, C
2		100.0, C	2		102.0, C
3		163.2, C	3		164.9, C
4	6.56, s	98.4, CH	4	6.40, d (2.2)	99.7, CH
5		165.1, C	5		166.1, C
6		113.4, C	6	6.28–6.29, m	106.4, CH
7	7.19, s	100.5, CH	7	3.26, dd (12.2)	35.5, $\text{CH}_2$
8		152.9, C	8	3.01, dd (3.1)	
9		132.1, C	9	5.45, dd (3.1)	80.5, CH
10/14	7.84–7.86, m	125.5, CH	10		130.4, C
11/13	7.42–7.49, m	129.1, CH	11	7.16–7.17, m	125.8, CH
12	7.42–7.49, m	130.2, CH	12	6.83, d (8.1)	116.0, CH
15		166.6, C	13		155.0, C
1'	3.09, dd (14.3, 4.2), 2.95, dd (14.2, 8.5)	31.6, $\text{CH}_2$	14	7.18–7.19, m	127.4, C
2'	4.26, dd (8.3, 4.0)	76.1, CH	15		128.4, CH
3'		147.6, C	1'	3.37, d (7.1)	170.0, C
4'	4.98, s, 4.87, s	110.8, $\text{CH}_2$	2'	5.31, t (7.2)	30.0, $\text{CH}_2$
5'	1.88, s	18.4, $\text{CH}_3$	3'		121.5, CH
5-OCH <sub>3</sub>	3.92, s	56.2, $\text{CH}_3$	4'	1.78, s	135.5, C
3-OH	11.42, s		5'	1.78, s	25.9, $\text{CH}_3$
			5-OCH <sub>3</sub>	3.84, s	18.1, $\text{CH}_3$
			3-OH	11.23, s	55.8, $\text{CH}_3$
			12-OH	5.26, s	

almost equal abundance. As shown in Fig. 4A, this deduction was further confirmed by chiral analysis of **1**, wherein two well-resolved peaks were observed in the chromatogram. Thus, the structure was finally identified and named as racemic cajanolactone B.

Compound **2** was obtained as a yellow oil. The HRESIMS data showed a protonated-molecular ion at  $m/z$  355.1539 [ $\text{M} + \text{H}$ ] $^+$  (calcd 355.1540), corresponding to a molecular formula of  $\text{C}_{21}\text{H}_{22}\text{O}_5$  with eleven degrees of unsaturation. The combination of 1D NMR (Table 1) and 2D NMR correlation signals (Fig. 2) showed the presence of a tetra-substituted benzene ring [ $\delta_{\text{H}}$  6.28–6.29 (1H, m), 6.40 (1H, d,  $J = 2.2$  Hz)], a tri-substituted

benzene ring [ $\delta_{\text{H}}$  6.83 (1H, d,  $J = 8.1$  Hz), 7.16–7.17 (1H, m), 7.18–7.19 (1H, m)], two linked aliphatic carbons ( $\delta_{\text{C}}$  35.5,  $\delta_{\text{C}}$  80.5), a typical isoprenyl moiety [ $\delta_{\text{C}}$  30.0, 121.5, 135.5, 25.9, 18.1], a chelated hydroxyl group [ $\delta_{\text{H}}$  11.23 (1H, s)] and a methoxy ( $\delta_{\text{C}}$  55.8).

Further close comparison of the NMR data of **2** with those of **1** suggested that **2** shared a similar isocoumarin skeleton. It was also found that the differences between them mainly revolved on the location of the free hydroxyl group and the isoprenyl moiety, as well as the absence of hydrogenation of the double bond at C-7/C-8 in **2**. This conclusion could be further



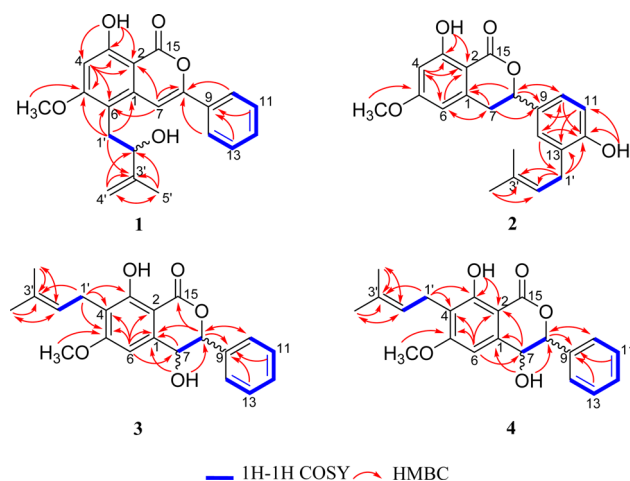


Fig. 2 Key  $^1\text{H}$ – $^1\text{H}$  COSY and HMBC correlations of compounds 1–4.

strengthened by the HMBC correlations from 12-OH to C-12, and  $\text{H}_2$  -1' to C-12, C-13.

Since the optical rotation of compound 2 was found being close to zero, it is speculated to be a pair of racemates and was then subjected to chiral analysis, in which two well-resolved peaks could be observed with almost identical abundance (Fig. 4B). Accordingly, the structure of compound 2 was established and named as racemic cajanolactone C.

Compound 3 was isolated as a yellowish oil. Its HRESIMS spectrum with the protonated molecular ion measured at  $m/z$  355.1541  $[\text{M} + \text{H}]^+$  (calcd 355.1540) suggested a molecular formula of  $\text{C}_{21}\text{H}_{22}\text{O}_5$ , corresponding to eleven indices of

hydrogen deficiency. Its IR showed characteristic resonance absorptions at 3379 and 1614  $\text{cm}^{-1}$ , accounting for the existence of hydroxyl and carboxyl functionalities, respectively.

The  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectra (Table 2) indicated the presence of a penta-substituted benzene ring [ $\delta_{\text{H}}$  6.71 (1H, s)], a monosubstituted benzene ring [ $\delta_{\text{H}}$  7.42–7.49 (5H, m)], a methoxy ( $\delta_{\text{C}}$  55.9), an isopentenyl group [ $\delta_{\text{H}}$  3.37 (2H, d,  $J = 7.1$  Hz), 5.20 (1H, t,  $J = 7.1$  Hz), 1.79 (3H, s), 1.68 (3H, s)], a chelated hydroxyl group [ $\delta_{\text{H}}$  11.44 (1H, s)], together with a free hydroxyl group [ $\delta_{\text{H}}$  2.09 (1H, s)]. Analyses of the 1D and 2D NMR data of 3 and 2 revealed their close resemblance except for the positions of the free hydroxyl group and the isoprenyl moiety in 3, which was further assigned by the observed HMBC cross-peaks of 7-OH to C-1 and C-8, and  $\text{H}_2$ -1'' to C-3 and C-4, respectively. Detailed inspection of 2D NMR (HSQC,  $^1\text{H}$ – $^1\text{H}$  COSY, HMBC) spectra established the planar structure of 3 as depicted in Fig. 1.

Similar to compound 2, the optical rotation of 3 was measured to be close to zero, implying its racemic character. Further chiral analysis evidenced that compound 3 is a mixture of two racemates with almost equal abundance (Fig. 4C). Moreover, the NOE correlations between H-7 and H-10/14 (Fig. 3), coupled with characteristic coupling constant  $J_{\text{H-7/H-8}} = 9.5$  Hz, which led to a trans configuration according to Karplus equation,<sup>14</sup> confirmed that 3 is a mixture of (7R\*, 8S\*) and (7S\*, 8R\*) racemates. Based on the above analysis, the structure of 3 was determined and named as racemic cajanolactone D1.

Compound 4 was also isolated as a yellowish oil, which exhibited an ion peak at  $m/z$  355.1531  $[\text{M} + \text{H}]^+$  (calcd 355.1540)

Table 2  $^1\text{H}$  (500 MHz) and  $^{13}\text{C}$  NMR (125 MHz) data of compounds 3 and 4,  $\delta$  in ppm

3 <sup>a</sup>			4 <sup>b</sup>		
No.	$\delta_{\text{H}}$ ( $J$ in Hz)	$\delta_{\text{C}}$ , type	No.	$\delta_{\text{H}}$ ( $J$ in Hz)	$\delta_{\text{C}}$ , type
1		140.5, C	1		141.0, C
2		100.2, C	2		100.6, C
3		161.0, C	3		159.9, C
4		117.0, C	4		116.1, C
5		164.0, C	5		162.7, C
6	6.71, s	98.9, CH	6	6.76, s	101.8, CH
7	4.91, d (9.5)	69.3, CH	7	4.88, dd (6.3, 1.6)	67.3, CH
8	5.22, d (9.5)	84.4, CH	8	5.74–5.75, m	82.2, CH
9		135.4, C	9		136.1, C
10/14	7.42–7.49, m	127.4, CH	10/14	7.60, d (7.5)	126.6, CH
11/13	7.42–7.49, m	129.5, CH	11/13	7.36–7.45, m	127.5, CH
12	7.42–7.49, m	129.0, CH	12	7.36–7.45, m	127.5, CH
15		168.8, C	15		169.2, C
1'	3.37, d (7.1)	21.8, $\text{CH}_2$	1'	3.35, d (7.3)	21.0, $\text{CH}_2$
2'	5.20, t (7.2)	121.6, CH	2'	5.21, t (7.3)	121.4, C
3'		132.2, C	3'		130.6, C
4'	1.79, s	17.8, $\text{CH}_3$	4'	1.77, s	16.4, $\text{CH}_3$
5'	1.68, s	25.8, $\text{CH}_3$	5'	1.65, s	24.5, $\text{CH}_3$
5-OCH <sub>3</sub>	3.92, s	55.9, $\text{CH}_3$	5-OCH <sub>3</sub>	3.96, s	55.1, $\text{CH}_3$
3-OH	11.44, s		3-OH	11.44, s	
7-OH	2.09, s		7-OH	4.66, d (6.3)	

<sup>a</sup> Recorded in  $\text{CDCl}_3$ . <sup>b</sup> Recorded in acetone- $\text{d}_6$ .



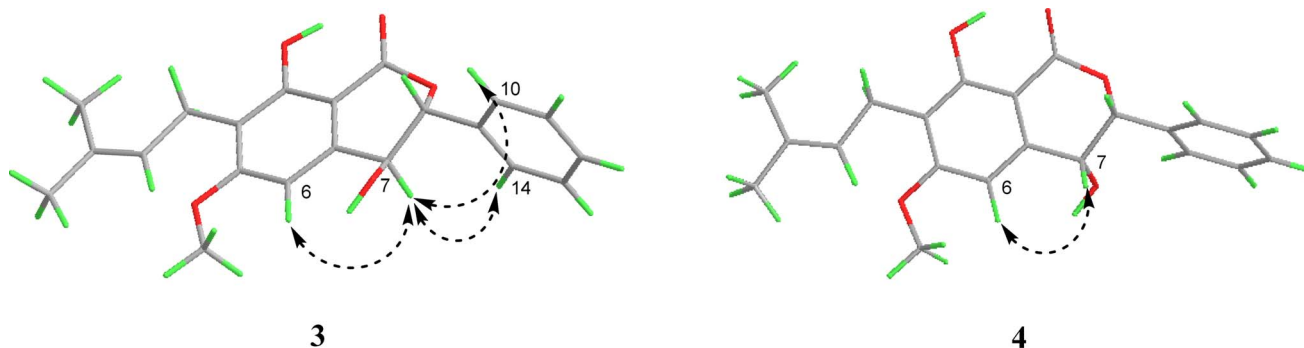


Fig. 3 Key NOESY correlations of compounds 3–4.

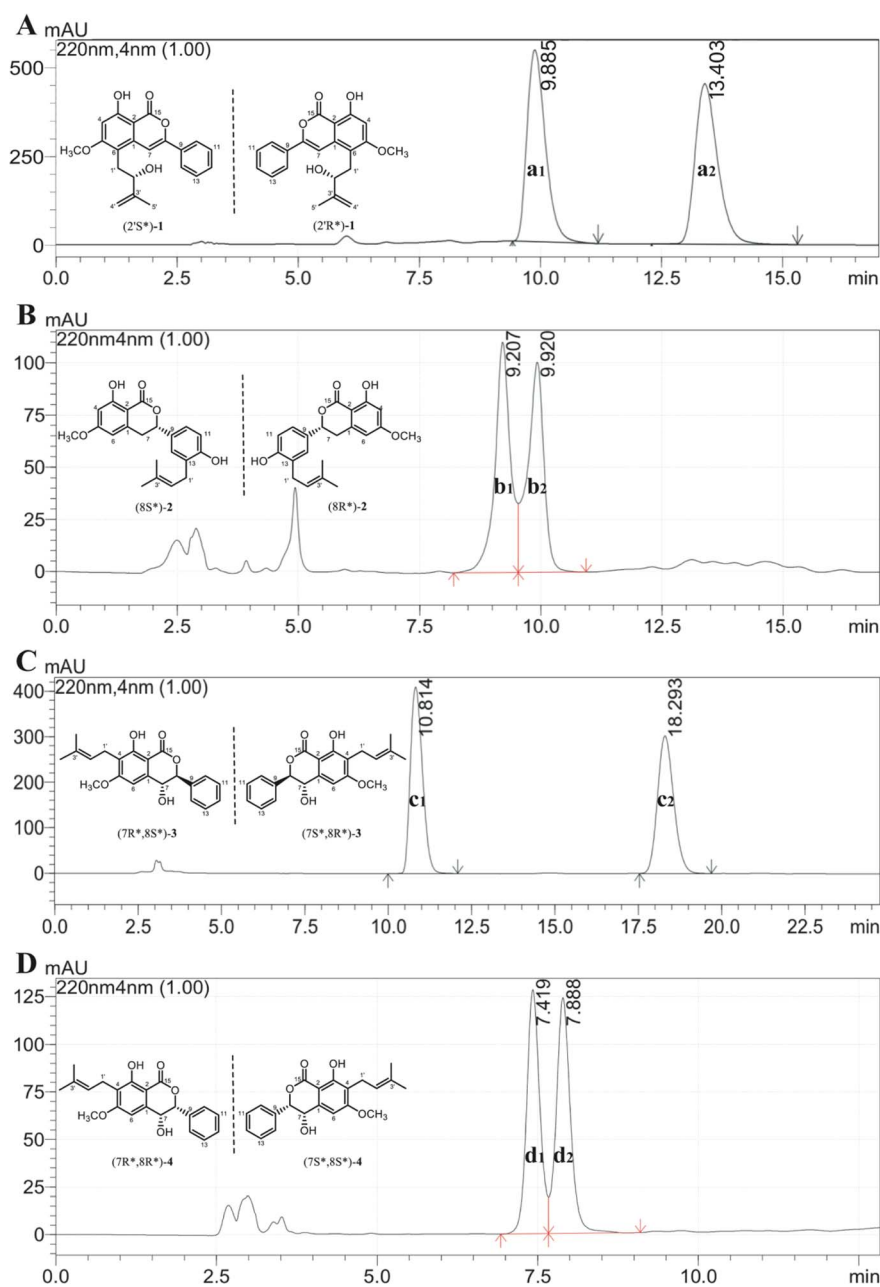
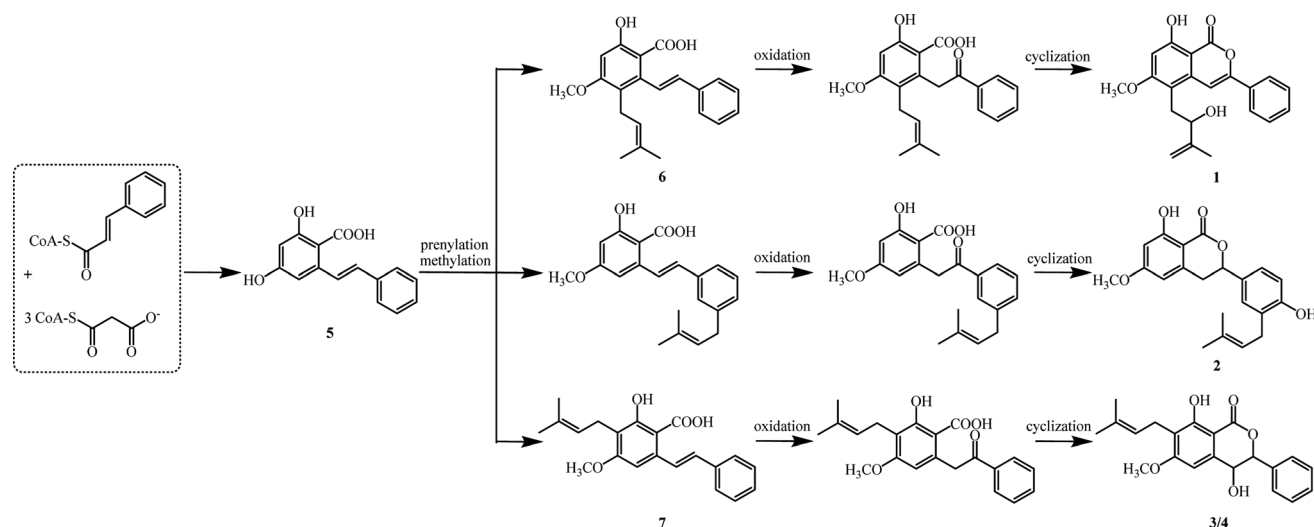


Fig. 4 Chiral analysis of compounds 1–4. (A) Relative peak area ratio of compound 1 was calculated as a1 : a2 = 49.4 : 50.6; (B) relative peak area ratio of compound 2 was calculated as b1 : b2 = 51.3 : 48.7; (C) relative peak area ratio of compound 3 was calculated as c1 : c2 = 49.2 : 50.8; (D) relative peak area ratio of compound 4 was calculated as d1 : d2 = 50.5 : 49.5.





Scheme 1 Possible biogenetic pathway to 1–4.

in HRESIMS spectrum with a positive mode, giving the molecular formula of  $C_{21}H_{22}O_5$  with eleven degrees of unsaturation.

The planar structure of **4** was determined to be the same as **3** based on a comparison of 1D and 2D NMR data. Similarly, compound **4** was considered as possible racemates owing to its optical rotation being close to zero, which was also verified *via* chiral analysis (Fig. 4D). However, there's a decent chance that compound **4** was a mixture of ( $7R^*$ ,  $8R^*$ ) and ( $7S^*$ ,  $8S^*$ ) racemic isomers since **3** was determined as a mixture of ( $7R^*$ ,  $8S^*$ ) and ( $7S^*$ ,  $8R^*$ ) racemates. The absent NOESY correlation of H-7 to H-10/14 (Fig. 3), along with the coupling constant of H-7 and H-8 ( $J_{H-7/H-8} = 1.6$  Hz) arising from the small spatic dihedral angle, were consistent with a *cis* configuration, namely a ( $7R^*$ ,  $8R^*$ ) or ( $7S^*$ ,  $8S^*$ ) relative configuration assignment. Collectively, compound **4** was elucidated and given a trivial name as racemic cajanolactone D2.

Biogenetically, isocoumarins are usually polyketide in origin and arise from the acetate–malonate pathway *via* cyclization reactions at the end(s) of a polyketide chain.<sup>15</sup> However, compounds **1–4** are more likely to originate biogenetically from the same bio-precursor named 2-hydroxy-4-methoxy-6-(2-phenylcinnyl)-benzoic acid (**5**), which is a stilbenoid produced by three malonyl-CoAs and one *p*-coumaroyl-CoA through stilbene synthase (STS).<sup>16</sup> The methylation and divergent prenylation of **5** lead to different intermediates, followed by critical oxidation and cyclization to form the lactone nucleus, which set the stage for generating **1–4**. The possible biosynthetic reaction was proposed as outlined in Scheme 1. Notably, bio-precursors **6–7** were also found as natural products from the leaves of *C. cajan*.<sup>17</sup> Compounds **1–4** represent the first members of a novel biogenetic pathway for the isocoumarins, which could be of great significance by providing a new viewpoint on isocoumarin metabolism in the plant kingdom.

### Biological evaluation

The bioassay was performed to evaluate the cytotoxicity for new compounds **1–4**, which were found to be devoid of detectable

cytotoxicity against HRT-18, Huh-7, and A549 cell lines even at high concentrations of 100  $\mu$ M.

## Experimental section

### General experimental procedures

The values of optical rotations were recorded using an MCP-500 spectropolarimeter (Anton Paar, Graz, Austria). The Applied Photophysics Chirascan were used to determine the ECD spectra of tested compounds. UV and IR data were acquired by a Shimadzu UV-2600 spectrophotometer (Shimadzu, Kyoto, Japan) and a Shimadzu IR Affinity-1 spectrometer (Shimadzu, Kyoto, Japan), respectively. HRESIMS spectra were obtained through a Thermo MAT95XP high resolution mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The measurement of 1D and 2D NMR data were collected on a Bruker Avance-500 spectrometer with aids of TMS as internal standard (Bruker, Fällanden, Switzerland). The 200–300 mesh silica gel (Qingdao Marine Chemical Inc., Qingdao, China) was used for column chromatography. TLC analysis was carried out on silica gel plates (Merck KGaA, Darmstadt, Germany). A Hitachi Primaide [Hitachi Instruments (Dalian) Co., Ltd.] equipped with a diode array detector (DAD) using a preparative YMC ODS  $C_{18}$  reverse column (20  $\times$  250 mm, 5  $\mu$ m) was used for semi-preparative HPLC separation. Organic solvents used were of analytical grade (Guangzhou Chemical Regents Company, Ltd., Guangzhou, China).

### Plant material

Leaves of *C. cajan* were collected from Honghe, Yunnan, P. R. China in August 2009 and authenticated by Professor Yunfei Deng of the South China Botanical Garden. A voucher specimen (SCIB 20210521) was deposited at the key laboratory of Natural Product Medicinal Chemistry, South China Botanical Garden, Chinese Academy of Sciences.





## Extraction and isolation

The mixture of air-dried, milled leaves of *C. cajan* (30.0 kg) were extracted with 95% EtOH (130 L) at room temperature for 72 h to provide a residue (5.0 kg), which was partitioned in *n*-hexane and EtOAc to obtain sequential extraction. The crude EtOAc extract was then subjected to a column chromatography over silica gel CC (100–200 mesh) and eluted with *n*-hexane/EtOAc (v/v 100:0 → 0:1) to yield fractions F<sub>1–11</sub>.

Fraction F<sub>6</sub> (20.0 g) was resolved into eight sub-fractions (F<sub>6-1</sub> to F<sub>6-8</sub>) by silica gel RP C<sub>18</sub> using MeOH/H<sub>2</sub>O (70 → 100%) as the eluent. F<sub>6-4</sub> (2.8 g) was applied to Sephadex LH-20 column chromatography, eluting with CHCl<sub>3</sub>/MeOH (v/v 1:3) to obtain further fractions (F<sub>6-4-1</sub> to F<sub>6-4-5</sub>). F<sub>6-4-5</sub> (1.5 g) was applied to silica gel CC (200–300 mesh) eluting with *n*-hexane/EtOAc (v/v 5:1) and then purified by RP-HPLC (YMC-Pack C<sub>18</sub>) with 60% acetonitrile in H<sub>2</sub>O to afford 3 (2.8 mg, *t*<sub>R</sub> = 32.0 min) and 4 (5.9 mg, *t*<sub>R</sub> = 35.0 min), respectively.

Fraction F<sub>10</sub> (39.8 g) was divided into nine further fractions (F<sub>10-1</sub> to F<sub>10-9</sub>) by column chromatography using silica gel RP C<sub>18</sub> under gradient conditions, using MeOH/H<sub>2</sub>O (40 → 100%) followed by MeOH/acetone (v/v 20:1 → 0:1) as mobile phase. F<sub>10-5</sub> (9.3 g) was isolated through Sephadex LH-20 column chromatography eluting with MeOH to acquire a number of sub-fractions (F<sub>10-5-1</sub> to F<sub>10-5-6</sub>), and F<sub>10-5-3</sub> was subjected to silica gel CC (200–300 mesh) to afford 2 (2.7 mg), eluting with *n*-hexane/EtOAc (v/v 5:1).

F<sub>10-6</sub> (2.2 g) was separated over a Sephadex LH-20 column chromatography eluting with CHCl<sub>3</sub>/MeOH (v/v 1:1) to acquire sub-fractions F<sub>10-6-1</sub> to F<sub>10-6-5</sub>. F<sub>10-6-2</sub> (246.0 mg) was subjected to Sephadex LH-20 column chromatography repeatedly and then purified with RP-HPLC (YMC-Pack C<sub>18</sub>) with 75% acetonitrile in H<sub>2</sub>O to yield 1 (4.7 mg, *t*<sub>R</sub> = 16.5 min).

Cajanolactone B (1) White powder; UV (MeOH) λ<sub>max</sub>/nm (log ε): 265 (3.09); IR ν<sub>max</sub>: 3374, 2920, 2851, 1668, 1622, 1576, 1472, 1456, 1242, 1204, 1148, 1103, 997, 764, 689 cm<sup>-1</sup>; <sup>1</sup>H (500 MHz) and <sup>13</sup>C (125 MHz) NMR data, see Table 1; ESIMS *m/z* 353 [M + H]<sup>+</sup>; HRESIMS *m/z* 353.1382 [M + H]<sup>+</sup> (for C<sub>21</sub>H<sub>21</sub>O<sub>5</sub><sup>+</sup>, calcd 353.1384).

Cajanolactone C (2) Yellow oil; UV (MeOH) λ<sub>max</sub>/nm (log ε): 267 (2.84), 302 (2.53); IR ν<sub>max</sub>: 3368, 2932, 1717, 1653, 1489, 1356, 1242, 1203, 1155, 1107, 1024, 949, 908, 795, 698, 567 cm<sup>-1</sup>; <sup>1</sup>H (500 MHz) and <sup>13</sup>C (125 MHz) NMR data, see Table 1; ESIMS *m/z* 355 [M + H]<sup>+</sup>; HRESIMS *m/z* 355.1539 [M + H]<sup>+</sup> (for C<sub>21</sub>H<sub>23</sub>O<sub>5</sub><sup>+</sup>, calcd 355.1540).

Cajanolactone D1 (3) Yellow oil; UV (MeOH) λ<sub>max</sub>/nm (log ε): 222 (3.46), 260 (2.95), 295 (2.50); IR ν<sub>max</sub>: 3379, 2940, 2847, 1728, 1614, 1466, 1456, 1339, 1242, 1196, 1126, 1090, 1016, 764, 719, 700, 631, 554 cm<sup>-1</sup>; <sup>1</sup>H (500 MHz) and <sup>13</sup>C (125 MHz) NMR data, see Table 2; ESIMS *m/z* 355 [M + H]<sup>+</sup>; HRESIMS *m/z* 355.1541 [M + H]<sup>+</sup> (for C<sub>21</sub>H<sub>23</sub>O<sub>5</sub><sup>+</sup>, calcd 355.1540).

Cajanolactone D2 (4) Yellow oil; UV (MeOH) λ<sub>max</sub>/nm (log ε): 222 (3.41), 260 (2.96), 295 (2.55); IR ν<sub>max</sub>: 3385, 2939, 2843, 1728, 1612, 1466, 1454, 1435, 1337, 1236, 1090, 1020, 764, 721, 700, 600, 575 cm<sup>-1</sup>; <sup>1</sup>H (500 MHz) and <sup>13</sup>C (125 MHz) NMR data, see Table 2; ESIMS *m/z* 355 [M + H]<sup>+</sup>; HRESIMS *m/z* 355.1531 [M + H]<sup>+</sup> (for C<sub>21</sub>H<sub>23</sub>O<sub>5</sub><sup>+</sup>, calcd 355.1540).

## Chiral analysis

Chiral analysis of compounds 1 and 4 was performed on a Shimadzu LC-15C HPLC with an EnantioPak® Y1 column (250 × 4.6 mm, 5 μm) using *n*-hexane/ethanol (v/v 85:15) and *n*-hexane/ethanol (v/v 90:10) as the eluent, respectively. Compound 2 was subjected to EnantioPak® R-C column (250 × 4.6 mm, 5 μm) eluting with *n*-hexane/ethanol (v/v 80:20), while 3 was analyzed using EnantioPak® Y3 column (250 × 4.6 mm, 5 μm) with *n*-hexane/ethanol (v/v 80:20) as the eluent. All these chiral columns were purchased from Guangzhou Research & Creativity Biotechnology Co., Ltd, China.

## Cytotoxicity assay

Compounds 1–4 were evaluated for their cytotoxic activity against HRT-18, Huh-7, and A549 cell lines using Cell Counting Kit-8 (CCK-8, Beyotime Biotechnology) according to the manufacturer's protocol. Doxorubicin was used as a positive control with IC<sub>50</sub> values of 1.4, 0.8, 1.2 μM for HRT-18, Huh-7, and A549 cell lines, respectively.

## Conclusion

Four new isocoumarins (1–4) were discovered during the investigation of *C. cajan*. Their structures were determined to be mixtures of racemic pairs, evident from chiral analysis, among which 3 and 4 share the same planer structure, which enriched the chemical diversity of the plant *C. cajan*. A plausible biogenetic pathway was proposed to account for the biosynthesis of compounds 1–4. However, further biological evaluation of these constituents is ongoing with an aim to explore the chemistry and biology of this plant.

## Conflicts of interest

The authors declare no conflict of interest that could have appeared to influence the work reported in this paper.

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