

HETEROCYCLES, Vol. 98, No. 3, 2019, pp. 444 - 450. © 2019 The Japan Institute of Heterocyclic Chemistry  
Received, 21st January, 2019, Accepted, 21st February, 2019, Published online, 13th March, 2019  
DOI: 10.3987/COM-19-14041

### THREE NEW ANTI-TOBACCO MOSAIC VIRUS PRENYL CHROMONE DERIVATIVES FROM *CASSIA NOMAME*

Yong Xu,<sup>1</sup> Qi-Li Mi,<sup>1</sup> Yin-Ke Li,<sup>1,2</sup> Wan-Li Zeng,<sup>1</sup> Qian Gao,<sup>1</sup> Chun-Man Song,<sup>1</sup> Hai-Tao Huang,<sup>1</sup> Hai-Ying Xiang,<sup>1</sup> Xue-Mei Li,<sup>1</sup> Guang-Yu Yang,<sup>1,2</sup> Qiu-Fen Hu,<sup>2\*</sup> and Jin Wang<sup>1\*</sup>

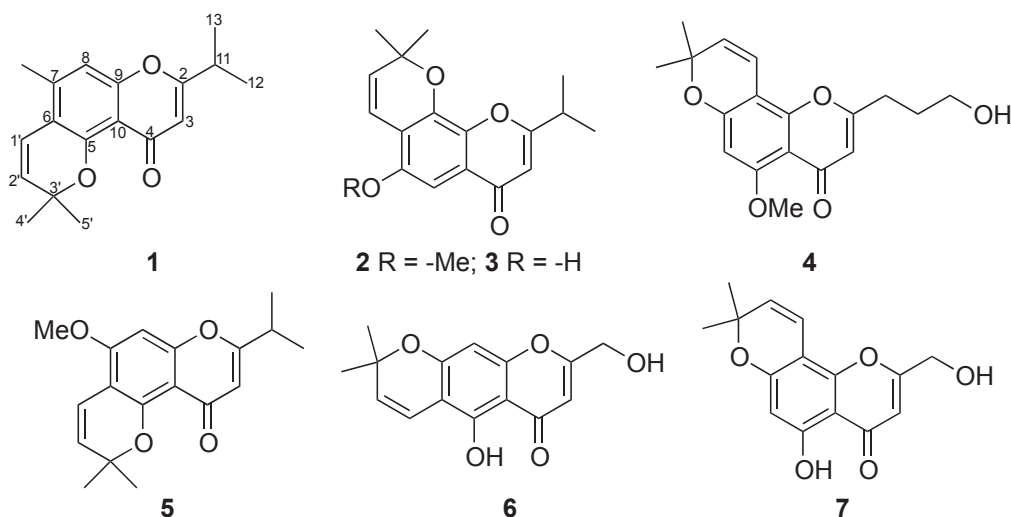
<sup>1</sup> Key Laboratory of Tobacco Chemistry of Yunnan Province, China Tobacco Yunnan Industrial Co., Ltd, 650231, Kunming, P. R. China. <sup>2</sup> Key Laboratory of Chemistry in Ethnic Medicinal Resources, Yunnan Minzu University, Kunming 650031, P. R. China. E-mail: jszxtg\_2015@163.com, huqiufena@aliyun.com

**Abstract** – Three new (**1-3**), together with four known (**4-7**) prenyl chromone derivatives were isolated from the whole plants of *Cassia nomame*. Their structures were elucidated by spectroscopic methods, including extensive 1D- and 2D-NMR techniques. Compounds **1-3** were evaluated for their anti-tobacco mosaic virus (Anti-TMV) activity. The results showed that compounds **1-3** showed potential anti-TMV activities with inhibition rates of 34.5%, 36.3%, and 57.2% at the concentration of 20  $\mu$ M, respectively. These rates are higher than that of positive control.

*Cassia nomame* is an annual herbs flowering plants of Cassia genus in the legume family, subfamily Caesalpinioideae.<sup>1</sup> It is a high biological yield plants and had been widely distributed in China.<sup>2</sup> The whole plants of *C. nomame* had widely been used as folk medication for long time in China for treatment of edema, nephritis, chronic constipation, cough, and phlegm.<sup>3</sup> The recent research also revealed that the flavonoids extract from *C. nomame* is a natural lipase inhibitor, which inhibits the lipase enzyme that breaks down fat for absorption.<sup>4</sup> Previous phytochemical studies of this plants have shown the presence of anthraquinones,<sup>5,6</sup> flavonoids,<sup>7-9</sup> chromones,<sup>10,11</sup> and the like.

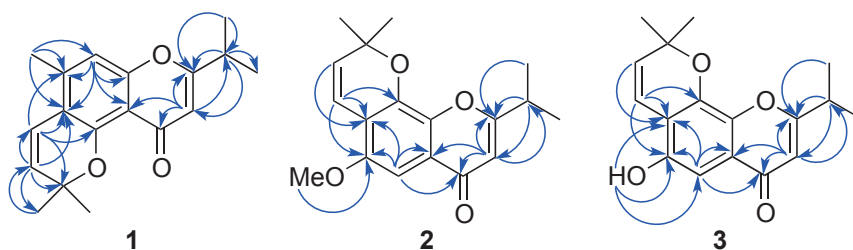
Chromone is a derivative of benzopyran with a substituted keto group on the pyran ring. These derivatives displayed a wide range of bioactivities, and received more and more attentions.<sup>12,13</sup> In our previous researches, some chromone derivatives from Cassia genus also founded to exhibit potential anti-TMV activity.<sup>14-17</sup> In our continuing efforts to identify bioactive natural products from Cassia genus, we now investigated the chemical constituents of the whole plant of *C. nomame*. This leads to the

isolation of three new (**1-3**) together with four known (**4-7**) prenyl chromone derivatives. The structures of **1-3** were elucidated by spectroscopic methods including extensive  $^1\text{D}$  and  $^2\text{D}$  NMR techniques. Compounds **1-3** were also evaluated for their anti-tobacco mosaic virus (anti-TMV) activities. This article deals with the isolation, structural elucidation and biological activities of these compounds.



**Figure 1.** The prenyl chromone derivatives from *Cassia nomame*

The air-dried whole plants of *C. nomame* were extracted with 95% methanol (MeOH), followed by repeated column chromatography on silica gel, Sephadex LH-20 and RP-18. Final purification by semi-preparative RP-HPLC afforded three new chromone derivatives, siamchromones R-T (**1-3**), together with four known chromone derivatives (**4-7**). The structures of compounds **1-7** were shown in Figure 1, and the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopic data of **1-3** were given in Table 1. The known compounds, compared with the literature, were identified as siamchromone F (**4**),<sup>16</sup> fistulachromone A (**5**),<sup>17</sup> greveichromenol (**6**),<sup>18,19</sup> and perforamone D (**7**).<sup>19</sup>



**Figure 2.** Key HMBC ( $\curvearrowright$ ) correlations of compounds **1-3**

Compound **1** was obtained as a yellow gum. It has the molecular formula  $\text{C}_{18}\text{H}_{20}\text{O}_3$  from HRESIMS ( $m/z$ : 307.1305  $[\text{M}+\text{Na}]^+$ , calcd 307.1310), with 9 degrees of unsaturation. The IR absorption bands indicated the presence of carbonyl ( $1660\text{ cm}^{-1}$ ), and aromatic ( $1615$ ,  $1560$ , and  $1462\text{ cm}^{-1}$ ) groups, and the UV

absorptions at 242, 272, and 350 nm suggested the existence of conjugated aromatic system. Its  $^1\text{H}$ ,  $^{13}\text{C}$ , and DEPT NMR spectroscopic data (Table 1) displayed signals for 18 carbons and 20 hydrogen atoms, corresponding to one chromone ring system (C-2~C-10) with two aromatic protons (H-3 and H-8),<sup>17</sup> one methyl group ( $\delta_{\text{C}}$  21.2 q,  $\delta_{\text{H}}$  2.00 s), one isopropyl moiety (-CH-(CH<sub>3</sub>)<sub>2</sub>; C-11~C-13, H-11 and H<sub>6</sub>-12,13),<sup>17,20</sup> and one 2,2-dimethyl-2*H*-pyran moiety (-CH=CH- C(CH<sub>3</sub>)<sub>2</sub>-O-; C-1'~C-5'; H-1', H-2', and H<sub>6</sub>-4',5').<sup>16</sup> The existence of a 2,2-dimethyl-2*H*-pyran moiety was confirmed by the HMBC correlations (Figure 2) from H-1' to C-5, C-6, C-7, C-2' and C-3', from H-2' to C-6, C-1', C-3', and C-4',5', and from H<sub>6</sub>-4',5' to C-3', and C-2'. Moreover, the chromone ring system was also supported by the HMBC correlations from H-3 to C-2, C-4, and C-10, from H-8 to C-9 and C-10.

**Table 1.**  $^{13}\text{C}$  and  $^1\text{H}$  NMR spectroscopic data for compounds **1-3** (125 and 500 MHz, in CDCl<sub>3</sub>)

No.	Compound 1		Compound 2		Compound 3	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$ ( <i>J</i> in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ ( <i>J</i> in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ ( <i>J</i> in Hz)
2	168.5 s		168.5 s		168.8 s	
3	105.8 d	6.29 s	106.2 d	6.28 s	106.0 d	6.27 s
4	179.2 s		180.1 s		180.3 s	
5	156.8 s		108.2 d	6.87 s	109.6 d	6.78 s
6	118.9 s		154.5 s		152.1 s	
7	144.1 s		116.9 s		118.6 s	
8	106.9 d	6.49 s	150.6 s		151.2 d	
9	155.2 s		144.7 s		145.6 s	
10	110.5 s		125.4 s		125.9 s	
11	33.4 d	2.61 m	33.5 d	2.63 m	33.4 d	2.68 m
12,13	19.8 q	1.09 (d) 6.8	20.0 q	1.08 (d) 6.8	19.9 q	1.09 (d) 6.8
1'	116.4 d	6.61 (d) 9.8	116.9 d	6.57 (d) 9.8	115.6 d	6.56 (d) 9.8
2'	128.6 d	5.67 (d) 9.8	128.7 d	5.65 (d) 9.8	128.4 d	5.63 (d) 9.8
3'	78.5 s		78.3 s		78.2 s	
4',5'	28.0 q	1.58 s	27.9 q	1.55 s	27.8 q	1.60 s
7-Me	21.2 q	2.00 s				
8-OMe			56.1 q	3.79 s		
8-OH						10.67 s

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopic data of **1** were similar to those of the known compound, fistulachromone A (**5**).<sup>17</sup> The chemical shift differences between them were resulted from the disappearance of a methoxy group signals and appearance of a methyl group signals ( $\delta_{\text{C}}$  21.2 q,  $\delta_{\text{H}}$  2.00 s). These evidences indicated that the methoxy group in **5** was converted into a methyl group in **1**. The HMBC correlations of H-11 ( $\delta_{\text{H}}$  2.61) with C-2 ( $\delta_{\text{C}}$  168.5) and C-3 ( $\delta_{\text{C}}$  105.8), of H<sub>6</sub>-12,13 ( $\delta_{\text{H}}$  1.09) with C-2 ( $\delta_{\text{C}}$  168.5), and of H-3 ( $\delta_{\text{H}}$  6.29) with C-11 ( $\delta_{\text{C}}$  33.4), indicated that the isopropyl moiety was attached

to C-2. The attachment of the methyl group at C-7 was supported by the HMBC correlations of the methyl proton ( $\delta_{\text{H}}$  2.00) with C-6 ( $\delta_{\text{C}}$  118.9), C-7 ( $\delta_{\text{C}}$  144.1), and C-8 ( $\delta_{\text{C}}$  106.9). Finally, long-range correlations from H-1' ( $\delta_{\text{H}}$  6.61) to C-5 ( $\delta_{\text{C}}$  156.8), C-6 ( $\delta_{\text{C}}$  118.9), and C-7 ( $\delta_{\text{C}}$  144.1), from H-2' ( $\delta_{\text{H}}$  5.67) to C-6 ( $\delta_{\text{C}}$  118.9) were observed. This led us to conclude that the 2,2-dimethyl-2*H*-pyran moiety was fused in an angular manner at C-6 and C-5. Accordingly, the structure of **1** was established, and given the trivial name of siamchromone R.

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopic data of siamchromone S (**2**) was obtained as a yellow gum with molecular formula  $\text{C}_{18}\text{H}_{20}\text{O}_4$  as determined by positive HRESI-MS ( $m/z$  323.1252  $[\text{M}+\text{Na}]^+$ ). Its  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were similar to those of **1**. The marked differences between them were due to the inexistence of a methyl group signals, appearance of a methoxy group signals ( $\delta_{\text{C}}$  56.1 s,  $\delta_{\text{H}}$  3.79 s), and the substituents group position variations in compound **2**. The isopropyl moiety located at C-2 was supported by the HMBC correlations (Figure 2) from H-11 to with C-2 and C-3, from H-12,13 to C-2, and from H-3 to C-11. The methoxy group located at C-6 was supported by the HMBC correlations from the methoxy proton ( $\delta_{\text{H}}$  3.79) to C-6 ( $\delta_{\text{C}}$  154.5). The 2,2-dimethyl-2*H*-pyran moiety located at C-7 and C-8 was supported by the HMBC correlations from H-1' to C-6, C-7, and C-8, from H-2' to C-7. Thus, the structure of **2** was determined as shown.

Siamchromone T (**3**) was also isolated as a yellow gum, and it gave a pseudomolecular ion peak at  $m/z$  309.1109  $[\text{M}+\text{Na}]^+$ , consistent with a molecular formula of  $\text{C}_{17}\text{H}_{18}\text{O}_4$ . Its  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopic data were also similar to those of **2**. The marked differences between them were due to the inexistence of a methoxy group signal, and appearance of a phenolic hydroxy proton ( $\delta_{\text{H}}$  10.67 s) in compound **3**. These change indicated that the methoxy group in **2** was replaced by a phenolic hydroxy group in compound **3**. The HMBC correlations (Figure 2) from phenolic hydroxy ( $\delta_{\text{H}}$  10.67) to C-5 ( $\delta_{\text{C}}$  109.6), C-6 ( $\delta_{\text{C}}$  152.1), and C-7 ( $\delta_{\text{C}}$  118.6) supported phenolic hydroxy group located at C-6. In addition, the other substituents positions also determined by the further analysis of its HMBC correlations. Thus, the structure of **3** was determined as shown.

Since certain chromones from *Cassia* genus exhibit potential anti-TMV activities,<sup>14-17</sup> compounds **1-3** were tested for their anti-TMV activity. The anti-TMV activity was tested using the half-leaf method. Ningnanmycin (a commercial product for plant disease in China) with inhibition rate of 34.8%, was used as a positive control.<sup>21,22</sup> The results revealed that compounds **1-3** showed high anti-TMV activity with inhibition rates of 34.5%, 36.3%, and 57.2% at the concentration of 20  $\mu\text{M}$ , respectively. These rates are higher than that of positive control.

Since the compound **3** exhibited higher inhibition rate for TMV, its  $\text{IC}_{50}$  values was also tested with ningnanmycin as the positive control. The results revealed that compound **3** exhibited the good activity with an  $\text{IC}_{50}$  value of 18.2  $\mu\text{M}$ ; the efficiency was higher than that of ningnanmycin (32.8  $\mu\text{M}$ ). In addition,

the protective effects of compound **3** on TMV were also evaluated by pretreating the tobacco plant with 20  $\mu$ M solutions of compounds or a solution of DMSO for 6 h before inoculation with TMV. The results showed that compound **3** showed protective effects to the host plants with the inhibition rate 59.4%. This results indicated that pretreatment with compound **3** could increase the resistance of the host plant to TMV infection.

**General Experimental Procedures.** UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. A Tenor 27 spectrophotometer was used for scanning IR spectra. 1D- and 2D-NMR spectroscopic data were recorded on a DRX-500 NMR spectrometer with TMS as internal standard. Chemical shifts ( $\delta$ ) are expressed in ppm with reference to the TMS signal. HRESIMS was performed on a VG Autospec-3000 spectrometer. Semi-preparative HPLC was performed on an Agilent 1260 preparative liquid chromatograph with Zorbax PrepHT GF (21.2 mm  $\times$  25 cm) or Venusil MP C<sub>18</sub> (20 mm  $\times$  25 cm) columns. Column chromatography was performed using silica gel (200-300 mesh, Qing-dao Marine Chemical, Inc., Qingdao, China), Lichroprep RP-18 gel (40-63  $\mu$ m, Merck, Darmstadt, Germany), Sephadex LH-20 (Sigma-Aldrich, Inc, USA), or MCI gel (75-150  $\mu$ m, Mitsubishi Chemical Corporation, Tokyo, Japan). Column fractions were monitored by TLC visualized by spraying with 5% H<sub>2</sub>SO<sub>4</sub> in ethanol and heating.

**Plant Material.** The whole plants of *Cassia nomame* (Sieb) Kitag, DC. were collected from Yuanjiang Prefecture, Yunnan province in September 2017. The species was identified by Prof. Chen Y. J. A voucher specimen (YNNI 17-9-83) was deposited in Key Laboratory of Chemistry in Ethnic Medicinal Resources, Yunnan Minzu University.

**Extraction and Isolation.** The air-dried samples (4.2 kg) were crushed to 30-50 mesh, and the powders were extracted with 95% MeOH (4 $\times$ 8 L) at room temperature and filtered. The filtrate was evaporated under reduced pressure, and the crude extract (362 g) was applied to a silica gel (150-200 mesh) column eluted with chloroform-methanol (CHCl<sub>3</sub>-MeOH) gradients (20:1, 9:1, 8:2, 7:3, 6:4, 5:5) to afford six fractions (A-F). Further separation of fraction B (9:1, 48.9 g) by silica gel column chromatography, eluted with CHCl<sub>3</sub>-acetone (1:0-1:2), yielded subfractions B1–B7. Subfraction B2 (9:1, 9.57 g) was loaded on another silica gel column using petroleum ether-ethyl acetate (EtOAc) elution, and then separated semi-preparative HPLC (66% MeOH-H<sub>2</sub>O, flow rate 20 mL/min) to afford **1** (14.5 mg) **2** (15.2 mg) and **5** (16.7 mg). Subfraction B3 (8:2, 6.24 g) was separated on the other silica gel column eluted by petroleum ether-EtOAc, followed by semi-preparative HPLC (58% MeOH-H<sub>2</sub>O, flow rate 20 mL/min) to give **3** (12.2 mg), and **4** (11.6 mg). Subfraction B4 (7:3, 7.51 g) was separated on another silica gel column eluted by petroleum ether-EtOAc, followed by semi-preparative HPLC (58% MeOH-H<sub>2</sub>O, flow rate 20 mL/min) to give **6** (16.4 mg), and **7** (15.0 mg).

**Anti-TMV Assays.** The anti-TMV activities were tested using the half-leaf method,<sup>21,22</sup> and

Ningnanmycin (2% water solution), a commercial product for plant disease in China, was used as a positive control. The virus was inhibited by mixing with the solution of tested compounds. After 30 min, the mixture was inoculated on the left side of the leaves of *Nicotiana glutinosa*, whereas the right side of the leaves was inoculated with the mixture of DMSO solution and the virus as control. The local lesion numbers were recorded 3-4 days after inoculation. Three repetitions were conducted for each compound. The inhibition rates were calculated according to the formula:

$$\text{inhibition rate (\%)} = [(C-T) / C] \times 100\%$$

where C is the average number of local lesions of the control and T is the average number of local lesions of the treatment. Ningnanmycin, a commercial virucide for plant disease in China, was used as a positive control.

Siamchromone R (**1**): C<sub>18</sub>H<sub>20</sub>O<sub>3</sub>, obtained as yellow gum; UV (MeOH) λ<sub>max</sub> (log ε) 212 (4.12), 242 (3.70), 272 (3.57), 350 (3.79) nm; IR (KBr) ν<sub>max</sub> 3122, 2950, 2864, 1660, 1615, 1560, 1462, 1154, 1068, 862, 779 cm<sup>-1</sup>; <sup>13</sup>C and <sup>1</sup>H NMR spectroscopic data (CDCl<sub>3</sub>, 125 and 500 MHz), see Table 1; positive ESIMS *m/z* 307 [M+H]<sup>+</sup>; HRESIMS *m/z* 307.1305 [M+Na]<sup>+</sup> (calcd for C<sub>18</sub>H<sub>20</sub>NaO<sub>3</sub>, 307.1310).

Siamchromone S (**2**): C<sub>18</sub>H<sub>20</sub>O<sub>4</sub>, obtained as yellow gum; UV (MeOH) λ<sub>max</sub> (log ε) 215 (4.22), 246 (3.75), 278 (3.52), 356 (3.82) nm; IR (KBr) ν<sub>max</sub> 3108, 2960, 2864, 1656, 1613, 1570, 1459, 1160, 1064, 855, 762 cm<sup>-1</sup>; <sup>13</sup>C and <sup>1</sup>H NMR spectroscopic data (CDCl<sub>3</sub>, 125 and 500 MHz), see Table 1; positive ESIMS *m/z* 323 [M+H]<sup>+</sup>; HRESIMS *m/z* 323.1252 [M+Na]<sup>+</sup> (calcd for C<sub>18</sub>H<sub>20</sub>NaO<sub>4</sub>, 323.1259).

Siamchromone T (**3**): C<sub>17</sub>H<sub>18</sub>O<sub>4</sub>, obtained as yellow gum; UV (MeOH) λ<sub>max</sub> (log ε) 214 (4.27), 243 (3.78), 275 (3.56), 352 (3.85) nm; IR (KBr) ν<sub>max</sub> 3410, 3087, 2957, 2846, 1654, 1614, 1562, 1439, 1155, 1072, 894, 785 cm<sup>-1</sup>; <sup>13</sup>C and <sup>1</sup>H NMR spectroscopic data (CDCl<sub>3</sub>, 125 and 500 MHz), see Table 1; positive ESIMS *m/z* 309 [M+H]<sup>+</sup>; HRESIMS *m/z* 309.1109 [M+Na]<sup>+</sup> (calcd for C<sub>17</sub>H<sub>18</sub>NaO<sub>4</sub>, 309.1103).

## ACKNOWLEDGEMENTS

This research was supported by the National Natural Science Foundation of China (No. 21562044), the Research Foundation of China Tobacco Yunnan Industrial Co., Ltd (No. 2017JC05), and the Young Academic and Technical Leader Rising Foundation of Yunnan Province (2017HB071).

## REFERENCES

1. D. L. Wu, *Flora of China*, Vol. 39, Beijing: Chinese Science Press, 1988, p 123.
2. J. Cai, J. Q. He, X. M. Wang, X. Shu, and L. Wu, *Chin. J. Agric. Sci. Bull.*, 2011, **27**, 142.
3. S. Q. Liu, Z. H. Liu, J. A. Huang, and Z. P. Shi, *Chin. Food Sci.*, 2005, **26**, 245.
4. N. Marchitto, F. Sindona, A. Fabrizio, M. Mauti, S. Andreozzi, S. Dalmaso, and G. Raimondi, *Minerva Cardioangiol.*, 2018, **66**, 124.

5. S. Kitanaka and M. Takido, *J. Nat. Prod.*, 1985, **48**, 849.
6. F. S. Eang, Y. L. Ding, and X. M. Deng, *Chin. Pharm. J.*, 2000, **35**, 37.
7. S. Kitanaka and M. Takido, *Phytochemistry*, 1992, **31**, 2927.
8. T. Konishi, K. Naitou, S. Kadowaki, Y. Takahara, and K. Yamamoto, *Biofactors*, 2004, **22**, 99.
9. T. Hatano, A. Yamashita, T. Hashimoto, H. Ito, N. Kubo, M. Yoshiyama, S. Shimura, Y. Itoh, T. Okuda, and T. Yoshida, *Phytochemistry*, 1997, **46**, 893.
10. S. Q. Liu, Z. H. Liu, J. A. Huang, and N. Tian, *Chin. J. Nat. Prod. Res. Dev.*, 2004, **13**, 244.
11. W. Dan, W.-n. Wang, B.-c. Qi, Q.-h. Zhang, and G.-y. Sun, *Chin. Herb. Med.*, 2013, **5**, 260.
12. C. Maicheen, N. Phosrithong, and J. Ungwitayatorn, *Med. Chem. Res.*, 2017, **26**, 662.
13. É. T. Oganessian, V. A. Tuskaev, and L. S. Sarkisov, *Pharm. Chem. J.*, 1994, **28**, 884.
14. Y. Li, Y. Meng, Y. Yang, Y. Qin, C. Xia, Y. Ye, X. Gao, and Q. Hu, *Phytochem. Lett.*, 2014, **10**, 46.
15. Q.-F. Hu, L.-M. Li, D.-L. Zhu, Z.-H. Yu, J.-B. Zhan, J. Lou, Y.-D. Wang, K. Zhou, M. Zhou, Y.-K. Li, and X.-M. Gao, *Heterocycles*, 2015, **91**, 1980.
16. Q.-F. Hu, B. Zhou, X.-M. Gao, L.-Y. Yang, L.-D. Shu, Y. Shen, G.-P. Li, C.-T. Che, and G.-Y. Yang, *J. Nat. Prod.*, 2012, **75**, 1909.
17. Q.-F. Hu, Y.-D. Wang, Z.-H. Yu, K. Zhou, W. Dong, M. Zhou, Y.-K. Li, X.-M. Gao, D.-L. Zhu, and Y.-Q. Ye, *Chem. Nat. Compd.*, 2017, **53**, 453.
18. F. M. Dean and M. L. Robinson, *Phytochemistry*, 1971, **10**, 3221.
19. S. Thadaniti, W. Archakunakorn, P. Tuntiwachwuttikul, and J. B. Bremner, *Chem. Pharm. Bull.*, 2006, **54**, 44.
20. S.-Z. Shang, W. Zhao, J.-G. Tang, J.-X. Pu, D.-L. Zhu, L. Yang, H.-D. Sun, G.-Y. Yang, and Y.-K. Chen, *Phytochem. Lett.*, 2016, **17**, 173.
21. M. Zhou, K. Zhou, X.-M. Gao, Z.-Y. Jiang, J.-J. Lv, Z.-H. Liu, G.-Y. Yang, M.-M. Miao, C.-T. Che, and Q.-F. Hu, *Org. Lett.*, 2015, **17**, 2638.
22. Q.-F. Hu, B. Zhou, J.-M. Huang, X.-M. Gao, L.-D. Shu, G.-Y. Yang, and C.-T. Che, *J. Nat. Prod.*, 2013, **76**, 292.