Three Cyclized Isoprenylated Flavonoids from the Roots and Rhizomes of *Sophora tonkinensis*

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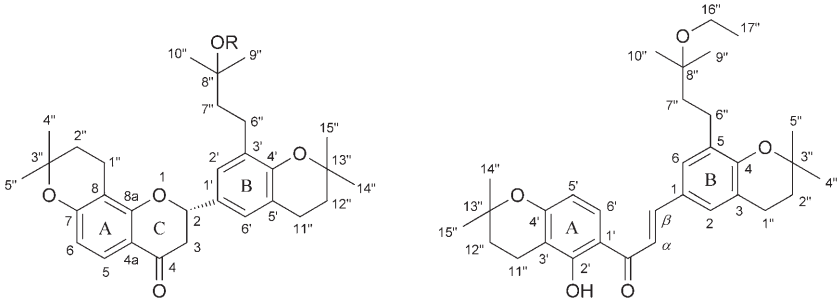
Two new flavanones, tonkinochromanes D (**1**) and E (**2**), and a new chalcone, tonkinochromane F (**3**), were isolated from the roots and rhizomes of *Sophora tonkinensis*. Their structures were elucidated by spectroscopic methods, including 2D-NMR and circular-dichroism (CD) techniques, in combination with HR-MS analysis. Compounds **1**–**3**, as well the previously reported tonkinochromanes A–C, were shown by LC-MS analysis to be 'artifacts' resulting from acid-catalyzed electrophilic cyclization of isoprenyl side chains during extraction.

Introduction. – As part of our continued search for cytotoxic phenolic agents from medicinal plants of the genus *Sophora* (Leguminosae) [1–3], a further phytochemical investigation of the flavonoid constituents of *S. tonkinensis* GAPNEP. (*S. subprostrata* CHUN et T. CHEN) was carried out. The roots and rhizomes of *S. tonkinensis* are commonly used in traditional Chinese medicine (TCM) as '*shan-dou-gen*' for the treatment of acute pharyngolaryngeal infections and sore throat [4]. Pharmacological studies showed that an isoprenylated flavanone isolated from this species, sophoranone, could inhibit cell growth and induce apoptosis in various cell lines from human solid tumors and in human leukemia U937 cells [5]. We previously reported three new prenylated flavonoids, tonkinochromanes A–C, from the roots and rhizomes of *S. tonkinensis* [3]. The present study describes the isolation of three further prenylated flavonoids, tonkinochromanes D–F (**1**–**3**), containing 2,2-dimethyldihydropyran rings.



1 R = H
2 R = Et

3

Isoprenylated flavonoids reported from *S. tonkinensis* contain either an unsaturated γ,γ-dimethylallyl side chain or a 2,2-dimethylpyran ring [6][7]. According to the literature [6], the γ,γ-dimethylallyl side chain in a flavonoid skeleton could cyclize with the neighboring OH function to form a chromane (= 3,4-dihydro-2*H*-1-benzopyran) when heated in alcoholic solution in the presence of HCl. Since in our present study the flavonoids were extracted with EtOH after the plant material had been pretreated with aq. 1% H_2SO_4 to separate the crude alkaloids first, we were suspicious that compounds **1**–**3**, as well as the previously isolated tonkinochromanes A–C [3], might be such reaction products. Here, we will show that this is, indeed, the case, and we will report, apart from the new structures, LC-MS studies concerning the determination of tonkinochromanes from plant extracts.

Results and Discussion. – 1. *Structure Elucidation*. The Et_2O-soluble fraction from the EtOH extract of the roots and rhizomes of *S. tonkinensis* was subjected to repeated chromatographic separation to afford three isoprenylated flavonoids, tonkinochromanes D–F (**1**–**3**)[1]), which were shown to be cyclization products arising from acid-catalyzed electrophilic addition (see below).

Compound **1**, obtained as colorless needles (m.p. 112–114°), was optically active ($[\alpha]_D^{20} = -30.0$ ($c = 0.10$, MeOH)). Its molecular formula was determined as $C_{30}H_{38}O_5$ by HR-ESI-MS (*m/z* 501.2614 ($[M+Na]^+$)). The IR spectrum showed absorption bands characteristic of a conjugated C=O group (1678 cm^{-1}) and an aromatic ring (1600, 1585, and 1479 cm^{-1}). The UV/VIS spectrum exhibited maximum absorptions at 312 nm (shoulder) and 285 nm, indicating a flavanone skeleton [8], which was further supported by three mutually coupled ^{1}H-NMR resonances (*Table 1*) at δ(H) 5.43 (*dd*, $J = 13.0$, 2.7, 1 H), 3.03 (*dd*, $J = 16.7$, 13.0 Hz, 1 H), and 2.69 (*dd*, $J = 16.7$, 2.7 Hz, 1 H), which were assigned to H–C(2), H–C(3a), and H–C(3b), respectively [8].

In addition, the ^{1}H-NMR spectrum of **1** showed the presence of a set of *ortho*-coupled aromatic signals [δ(H) 7.61, 6.45 (2*d*, $J = 8.7$ Hz each, 2×1 H)] and a set of *meta*-coupled ones [δ(H) 7.17, 7.12 (2*d*, $J = 1.4$ Hz each, 2×1 H)], as well as two sets of resonances due to two 2,2-dimethyldihydropyran groups fused to rings *A* and *B*, respectively, of a flavanone, with signals at δ(H) 2.63–2.70 (*m*, 2 H), 1.80–1.86 (*m*, 2 H), 1.34, 1.32 (2*s*, 2×3 H); and at 2.82 (*t*, $J = 6.8$ Hz, 2 H), 1.84 (*t*, $J = 6.8$ Hz, 2 H), and 1.35 (*s*, 6 H) [9]. The signals at δ(H) 2.63–2.70 (*m*, 2 H) and 1.70–1.73 (*m*, 2 H), and a signal at δ(H) 1.24 (*s*, 6 H) could be assigned with the aid of an HMBC experiment (*Fig. 1*) to the two pairs of CH_2 and Me groups of a 3-hydroxy-3-methylbutyl substituent fused to ring *B* [10]. This was confirmed by the EI-MS fragment ions of **1** at *m/z* 205 ($[A_1+H]^+$) and 274 ($B_1^{+\bullet}$), arising from *retro-Diels–Alder* cleavage of the flavanone *C*-ring [11].

The ^{13}C-NMR spectrum of **1** contained signals for a C=O group (δ(C) 191.2), three oxygenated aromatic C-atoms (δ(C) 162.1, 161.5, 153.1), as well as the flavanone atoms C(2) and C(3) (δ(C) 81.1, 44.9, resp.) (*Table 2*). The aromatic signal at δ(H) 7.61, which correlated with C(4) (δ(C) 191.2), C(7) (161.5), and C(8a) (162.1) in the HMBC spectrum, could be assigned to H–C(5). Since it coupled with the *ortho* H–C(6) [δ(H)

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[1]) Arbitrary atom numbering, adapted from trivial flavonoid numbering. For systematic names, see *Exper. Part.*

Table 1. *^{1}H-NMR Data of* **1**–**3**. In (D_6)acetone; δ in ppm, J in Hz. Arbitrary atom numbering.

Atom	**1**[a])	**2**[a])	**3**[b])
H–C(2)	5.43 (*dd*, *J* = 13.0, 2.7)	5.39 (*dd*, *J* = 13.1, 2.6)	7.43 (br. *s*)
H–C(3a)	3.03 (*dd*, *J* = 16.7, 13.0)	3.00 (*dd*, *J* = 16.7, 13.1)	–
H–C(3b)	2.69 (*dd*, *J* = 16.7, 2.7)	2.67 (*dd*, *J* = 16.7, 2.6)	–
H–C(5)	7.61 (*d*, *J* = 8.7)	7.61 (*d*, *J* = 8.8)	–
H–C(6)	6.45 (*d*, *J* = 8.7)	6.44 (*d*, *J* = 8.8)	7.51 (br. *s*)
H–C(α)	–	–	7.76 (*AB q*, *J* = 15.2)
H–C(β)	–	–	7.81 (*AB q*, *J* = 15.2)
H–C(2′)	7.17 (*d*, *J* = 1.4)	7.17 (br. *s*)	–
OH–C(2′)	–	–	14.18 (*s*)
H–C(5′)	–	–	6.33 (*d*, *J* = 8.9)
H–C(6′)	7.12 (*d*, *J* = 1.4)	7.10 (br. *s*)	7.94 (*d*, *J* = 8.9)
CH_2(1″)	2.63–2.70 (*m*)	2.62–2.69 (*m*)	2.84 (*t*, *J* = 6.8)
CH_2(2″)	1.80–1.86 (*m*)	1.77–1.84 (*m*)	1.86 (*t*, *J* = 6.8)
Me(4″)	1.32 (*s*)	1.31 (*s*)	1.36 (*s*)
Me(5″)	1.34 (*s*)	1.33 (*s*)	1.36 (*s*)
CH_2(6″)	2.63–2.70 (*m*)	2.59–2.63 (*m*)	2.59–2.63 (*m*)
CH_2(7″)	1.70–1.73 (*m*)	1.70–1.73 (*m*)	1.69–1.73 (*m*)
Me(9″)	1.24 (*s*)	1.20 (*s*)	1.20 (*s*)
Me(10″)	1.24 (*s*)	1.20 (*s*)	1.20 (*s*)
CH_2(11″)	2.82 (*t*, *J* = 6.8)	2.81 (*t*, *J* = 6.7)	2.67 (*t*, *J* = 6.7)
CH_2(12″)	1.84 (*t*, *J* = 6.8)	1.83 (*t*, *J* = 6.7)	1.84 (*t*, *J* = 6.7)
Me(14″)	1.35 (*s*)	1.35 (*s*)	1.34 (*s*)
Me(15″)	1.35 (*s*)	1.35 (*s*)	1.34 (*s*)
CH_2(16″)	–	3.45 (*q J* = 7.0)	3.46 (*q J* = 7.0)
Me(17″)	–	1.13 (*t*, *J* = 7.0)	1.13 (*t*, *J* = 7.0)

[a]) At 500 MHz. [b]) At 400 MHz.

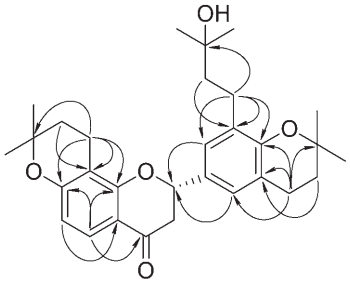

Fig. 1. *Selected HMBC correlations of* **1**

6.45 (*d*, *J* = 8.7 Hz, 1 H)], the fusion site of the 2,2-dimethyldihydropyran group at ring *A* was established at C(7) and C(8). Further evidence for this deduction was provided by a HMBC experiment, in which the CH_2(2″) and CH_2(1″) groups (δ(H) 1.80–1.86 and 2.63–2.70, resp.) both correlated with C(8) (δ(C) 110.4); the latter CH_2 H-atoms also coupled with C(7) and C(8a) (δ(C) 161.5 and 162.1, resp.).

The fusion site of the dihydropyran moiety at ring *B*, *i.e.*, at C(4′) and C(5′), was deduced by correlations of CH_2(12″) (δ(H) 1.84) with C(5′) (δ(C) 121.7), and of

Table 2. *^{13}C-NMR Data of* **1**–**3**. In (D_6)acetone; δ in ppm. Arbitrary numbering.

Atom	**1**[a])	**2**[a])	**3**[b])	Atom	**1**[a])	**2**[a])	**3**[b])
C(1)	–	–	127.2	C(6′)	126.6	126.6	129.79
C(2)	81.1	81.0	129.75	C(1″)	17.9	17.9	23.1
C(3)	44.9	44.9	122.1	C(2″)	32.7	32.7	33.1
C(4)	191.2	191.1	155.5	C(3″)	76.4	76.4	75.8
C(4a)	115.1	115.1	–	C(4″)	26.9	26.9	27.2
C(5)	126.4	126.4	132.6	C(5″)	27.57	27.58	27.2
C(6)	112.6	112.5	129.3	C(6″)	26.7	26.3	25.8
C=O	–	–	193.0	C(7″)	45.5	41.3	40.9
C(α)	–	–	118.1	C(8″)	70.5	74.9	74.5
C(β)	–	–	145.6	C(9″)	30.0	26.6	26.2
C(7)	161.5	161.4	–	C(10″)	30.0	26.6	26.2
C(8)	110.4	110.3	–	C(11″)	23.7	23.7	17.0
C(8a)	162.1	162.0	–	C(12″)	33.7	33.7	32.3
C(1′)	131.4	131.3	113.7	C(13″)	75.3	75.3	76.4
C(2′)	127.0	127.1	165.0	C(14″)	27.63	27.63	26.9
C(3′)	132.4	132.2	109.9	C(15″)	27.61	27.7	26.9
C(4′)	153.1	153.1	161.5	C(16″)	–	57.1	56.7
C(5′)	121.7	121.7	109.7	C(17″)	–	17.0	16.6

[a]) At 125 MHz. [b]) At 100 MHz.

CH_2(11′) (δ(H) 2.82) with C(4′), C(5′), and C(6′) (δ(C) 153.1, 121.7, and 126.6, resp.); also, H–C(6′) (δ(H) 7.12) correlated with C(2) (δ(C) 81.1) in the HMBC spectrum. In addition, cross-peaks of CH_2(7″) (δ(H) 1.70–1.73) with C(3′) (δ(C) 132.4), and those of CH_2(6″) (δ(H) 2.63–2.70) with C(4′), C(3′), and C(2′) (δ(C) 153.1, 132.4, and 127.0, resp.) were observed. The signal at δ(H) 7.17 (H–C(2′)), correlating with C(2) (δ(C) 81.1), indicated that the 3-hydroxy-3-methylbutyl substituent at ring *B* was connected to C(3′) (*Fig. 1*). With the aid of HMBC and HMQC experiments, all ^{1}H- and ^{13}C-NMR signals could, thus, be fully assigned.

The absolute configuration of **1** at C(2) was determined as (*S*) from the circular-dichroism (CD) spectrum, which showed a positive *Cotton* effect at 333 nm, and a negative one at 304 nm [12]. From these data, the structure of **1** was determined as (2*S*)-2,3,9,10-tetrahydro-2-[3,4-dihydro-8-(3-hydroxy-3-methylbutyl)-2,2-dimethyl-2*H*-chromen-6-yl]-8,8-dimethyl-4*H*,8*H*-pyrano[2,3-*f*]chromen-4-one, and named *tonkinochromane D*.

Compound **2**, obtained as a colorless oil, was optically active ($[\alpha]_D^{20} = -47.5$ (c = 0.30, MeOH)). HR-ESI-MS Analysis showed the $[M+Na]^+$ peak at *m/z* 529.2927, in accord with the molecular formula $C_{32}H_{42}O_5$. By comparing the NMR data of **2** (*Tables 1* and *2*) with those of **1**, it was inferred that the OH group of the 3-hydroxy-3-methylbutyl substituent in **1** was replaced with an EtO group in **2**. The NMR data of **2** were identical to those of **1**, except for the additional EtO signals [δ(H) 3.45 (q, J = 7.0 Hz, 2 H), 1.13 (t, J = 7.0 Hz, 3 H); δ(C) 57.1, 17.0]. This deduction was further substantiated by different molecular-ion masses ($\Delta m/z = 28$ (C_2H_4)). In the ^{13}C-NMR spectrum of **2** (*Table 2*), the noticeable downfield shift of C(8″) ($\Delta\delta$(C) = +4.4) as well as the upfield shifts of C(7″) ($\Delta\delta$(C) = –4.2 ppm), C(9″) ($\Delta\delta$(C) = –3.4 ppm), and

C(10″) ($\Delta\delta$(C) = −3.4 ppm) suggested that the EtO group was attached to C(8″). This was verified by HMBC correlations between the CH_2 H-atoms of the EtO group at δ(H) 3.45 and the O-connected quaternary C-atom at δ(C) 74.9 (C(8″)). The structure of **2** was further confirmed by analyses of HMQC and HMBC spectra.

The absolute configuration of **2** at C(2) was determined as (*S*) according to the CD spectrum, which showed a positive *Cotton* effect at 333 nm, and a negative one at 303 nm [12]. Thus, the structure of **2** was elucidated as (2*S*)-2,3,9,10-tetrahydro-2-[3,4-dihydro-8-(3-ethoxy-3-methylbutyl)-2,2-dimethyl-2*H*-chromen-6-yl]-8,8-dimethyl-4*H*,8*H*-pyrano[2,3-*f*]chromen-4-one, and named *tonkinochromane E*.

Compound **3**, obtained as yellow oil, was assigned the molecular formula $C_{32}H_{42}O_5$, based on HR-ESI-MS analysis (*m/z* 507.3105 ($[M + H]^+$)). The compound showed a positive reaction with the $FeCl_3$ reagent, indicative of a phenolic moiety. Its IR spectrum exhibited absorption bands characteristic of an OH group (3441 cm^{-1}), an α,β-unsaturated C=O group (1629 cm^{-1}), and aromatic rings (1588, 1564, 1469 cm^{-1}). The UV/VIS maxima at 381 nm and 245 nm (shoulder), as well as a pair of *AB*-type ^{1}H-NMR signals centered at δ(H) 7.76 and 7.81 (*AB*-type *q*, *J* = 15.2 Hz, 1 H each) suggested the presence of a chalcone skeleton [8].

The ^{1}H-NMR spectrum of **3** (*Table 1*) exhibited signals for two 2,2-dimethyldihydropyran groups [δ(H) 2.84 (*t*, *J* = 6.8 Hz, 2 H), 1.86 (*t*, *J* = 6.8 Hz, 2 H), 1.36 (*s*, 6 H); and δ(H) 2.67 (*t*, *J* = 6.7 Hz, 2 H), 1.84 (*t*, *J* = 6.7 Hz, 2 H), 1.34 (*s*, 6 H)] and of a 3-ethoxy-3-methylbutyl side chain [δ(H) 3.46 (*q*, *J* = 7.0 Hz, 2 H), 2.59–2.63 (*m*, 2 H), 1.69–1.73 (*m*, 2 H), 1.20 (*s*, 6 H), 1.13 (*t*, *J* = 7.0 Hz, 3 H)], similar to that in **2**. It also contained a chelated OH group [δ(H) 14.18 (*s*, 1 H)], a pair of *ortho*-coupled aromatic H-atoms [δ(H) 7.94, 6.33 (2*d*, *J* = 8.9 Hz each, 2 × 1 H)], and a set of *meta*-coupled ones [δ(H) 7.51, 7.43 (2 br. *s*, 2 × 1 H)]. The aromatic signal at δ(H) 7.94 was assigned to H–C(6′) due to its downfield chemical shift, resulting from the deshielding effect of the neighboring C=O group, and its HMBC correlations with δ(C) 193.0 (C=O), 165.0 (C(2′)), and 161.5 (C(4′)). Since it coupled with H–C(5′) [δ(H) 6.33 (*d*, *J* = 8.9 Hz, 1 H)], the 2,2-dimethyldihydropyran group at ring *A* was determined to be at C(3′) and C(4′), which was corroborated by the following HMBC correlations: δ(H) 1.84 (CH_2(12″)) with C(3′) (δ(C) 109.9); δ(H) 2.67 (CH_2(11″)) with C(2′) (δ(C) 165.0), C(3′) (δ(C) 109.9), and C(4′) (δ(C) 161.5); and 2′-OH (δ(H) 14.18) with C(1′) (δ(C) 113.7), C(2′) (δ(C) 165.0), and C(3′) (δ(C) 109.9). As to ring *B*, the two *meta*-coupled aromatic signals at δ(H) 7.51 and 7.43 both correlated with C_β at δ(C) 145.6 in the HMBC spectrum, indicating that the substituents were located at C(3), C(4), and C(5). The correlations of CH_2(6″) at δ(H) 2.59–2.63 with C(4) (δ(C) 155.5), C(5) (δ(C) 132.6), and C(6) (δ(C) 129.3), and of CH_2(1″) at δ(H) 2.84 with C(2) (δ(C) 129.75), C(3) (δ(C) 122.1), and C(4) (δ(C) 155.5) further suggested that the 3-ethoxy-3-methylbutyl chain was connected to C(5), and that the other dihydropyran group was fused to C(3) and C(4), resp. (*Fig. 2*). With the aid of further HMBC and HMQC experiments, all ^{1}H- and ^{13}C-NMR signals could be fully assigned.

Thus, the structure of compound **3** was established as (2*E*)-3-[3,4-dihydro-8-(3-ethoxy-3-methylbutyl)-2,2-dimethyl-2*H*-chromen-6-yl]-1-(3,4-dihydro-5-hydroxy-2,2-dimethyl-2*H*-chromen-6-yl)prop-2-en-1-one, and named *tonkinochromane F.*

2. *Artifact Formation*. To establish whether tonkinochromanes A–F were 'artifacts' produced during plant treatment with 1% H_2SO_4 and extraction with EtOH (for the

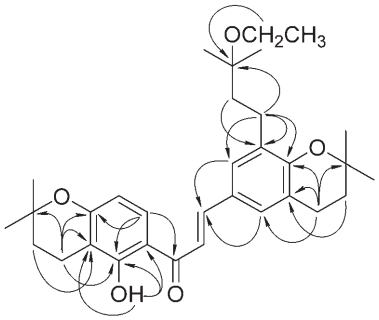

Fig. 2. *Selected HMBC correlations of* **3**

separation of crude alkaloids), we performed a separate extraction with EtOH only. The EtOH extract and the extract used for the isolation of **1**–**3** were then analyzed by LC-MS. We found that these isoprenylated flavonoids could *not* be detected in the EtOH extract obtained without preliminary acidic treatment, whereas in the acid-treated EtOH extract used in the present study tonkinochromanes A–F were all detected in relatively high yields, their retention times and quasi-molecular ion peaks ($[M + H]^+$) being identical to those of the corresponding reference flavonoids (see *Fig. 3* and *Exper. Part*).

From these results, we conclude that the isolated tonkinochromanes A–F are, indeed, artifacts formed by acid-catalyzed electrophilic cyclization of isoprenyl side chains. Thus, tonkinochromanes A, B, D, and E might be derived from sophoranone [6], and tonkinochromanes C and F could stem from sophoradin [6] and sophoradochromene [7], respectively. All of these precursors have been reported to be present in the MeOH extract of *S. tonkinensis* [6][7].

3. *Biological Studies.* The isolated tonkinochromanes D (**1**) and E (**2**)[2]) were tested *in vitro* against a panel of human tumor cell lines, including human lung carcinoma (A549), ovarian carcinoma (1A9), breast adenocarcinoma (MCF-7), epidermoid carcinoma of the nasopharynx (KB), as well as its drug-resistant variant (KB-Vin). However, both compounds were inactive ($IC_{50} > 20$ μg/ml). These results are in agreement with the structure–activity relationship previously reported for this type of compounds [2][3].

Experimental Part

1. *General.* Petroleum ether (PE) for chromatography had a boiling-point range of 60–90°. Column chromatography (CC): silica gel *H* (200–300 mesh; *Qingdao*, China). Anal. (0.25 mm) and prep. (0.50 mm) TLC: precoated silica-gel plates GF_{254} (10–40 μm; *Yantai*, China), detection under UV light and by staining with 10% aq. H_2SO_4 followed by heating. Melting points (m.p.): *XT-4* micro-melting-point apparatus; uncorrected. UV Spectra: *Shimadzu UV-260* UV/VIS spectrophotometer; λ_{max} (log ε) in nm. Optical rotations: *Jasco P-1020* digital polarimeter. CD Spectra: *Jasco J-715* spectropolarimeter; λ ($[\theta]$) in nm. IR Spectra: *Avatar 360 FT-IR* spectrophotometer, with KBr pellets or in CH_2Cl_2 soln.; in cm^{-1}. NMR Spectra: *Bruker DRX-400* or *-500* spectrometers, at 400 or 500 MHz (^1H), and at 100 or 125 MHz (^{13}C), resp., in (D_6)acetone soln. at r.t.; δ in ppm rel. to Me_4Si, *J* in Hz. EI-MS: *Hewlett-Packard*

[2]) Tonkinochromane F (**3**) could not be tested due to the limited amount of sample obtained.

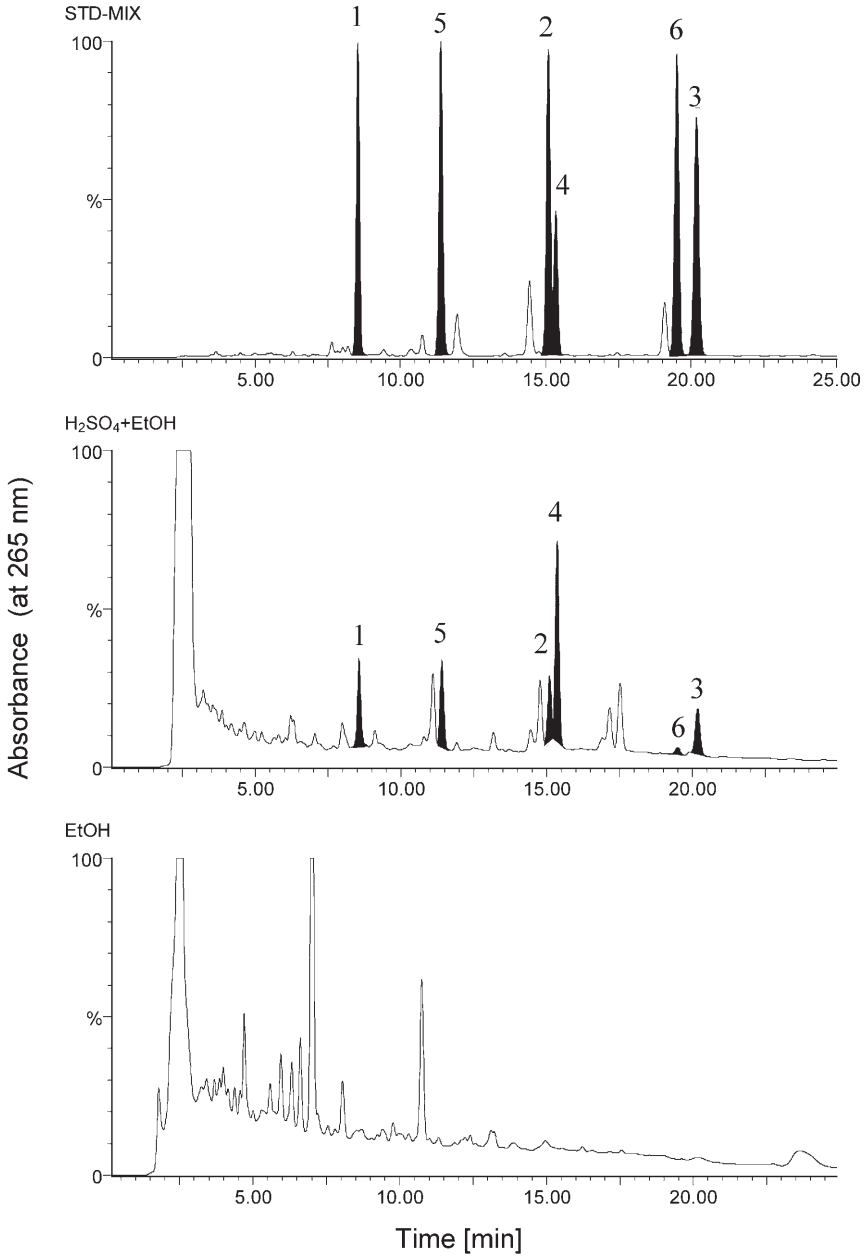

Fig. 3. *HPLC Chromatograms of flavonoid-standard mixture* (*a*), *EtOH extract of S. tonkinensis after* (*b*) *and without* (*c*) *aq. H_2SO_4 pretreatment.* Full-scale absorbances *A* (at 265 nm): 0.5, 3.3, and 2.2 a.u. for *a*)–*c*), resp. *Peaks 1–6* represent tonkinochromanes D, E, F, A, B, and C, resp.

5989A mass spectrometer; in *m/z* (rel. %). HR-ESI-MS: *AB-QSTAR-Pulsar* mass spectrometer. LC-MS: *Waters Alliance HT 2795-ZQ2000* system.

2. *Plant Material.* The roots and rhizomes of *Sophora tonkinensis* GAPNEP. were purchased from *Huayu Materia Medica Co., Ltd.*, Shanghai, in March 2000. The plant was authenticated by one of the authors (*D. C.*). A voucher specimen (SDG-SH-0003) was deposited at the Herbarium of Materia Medica, Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai, P. R. China.

3. *Extraction and Isolation.* The pulverized roots and rhizomes of *S. tonkinensis* (9 kg) were extracted with 1% (*v/v*) aq. H_2SO_4 (4 × 15 l) at r.t. overnight to afford the total-alkaloid fraction (120 g). After solvent removal, the residue was air-dried at r.t. and macerated with 95% EtOH (6 × 12 l) at r.t. overnight. The EtOH extract was evaporated to dryness *in vacuo* at 50° to afford a residue (1.5 kg), which was suspended in H_2O (2.5 l) and partitioned with Et_2O (6 × 3 l). The Et_2O-soluble fraction (220 g) was subjected to CC (SiO_2, PE/AcOEt 100:1, 50:1, 30:1, 20:1, 10:1, 5:1, 2:1, 1:1, 0:1) to afford nine fractions (*Fr. 1–Fr. 9*). *Fr. 2* (10.05 g) was subjected to CC (SiO_2; PE/Me_2CO 80:1): to yield five subfractions: *Fr. 2A–Fr. 2E*. *Fr. 2B* (2.50 g) was subjected to CC (SiO_2; PE/Et_2O 20:1) and then to prep. TLC (SiO_2; PE/AcOEt 92:8) to afford **3** (3 mg). *Fr. 6* (12.60 g) was subjected to CC (SiO_2; PE/$CHCl_3$/Me_2CO 50:50:3) and then to prep. TLC (SiO_2; PE/$CHCl_3$/Me_2CO 10:10:3) to yield **1** (25 mg). The residue of the latter separation was further subjected to CC (SiO_2; $CHCl_3$/Me_2CO 50:1) and then to prep. TLC (SiO_2; C_6H_6/Me_2CO 9:1) to afford **2** (50 mg).

4. *Compound Characterization.* 4.1. *Tonkinochromane D* (=*(2*S*)-2,3,9,10-Tetrahydro-2-[3,4-dihydro-8-(3-hydroxy-3-methylbutyl)-2,2-dimethyl-2*H*-chromen-6-yl]-8,8-dimethyl-4*H*,8*H*-pyrano[2,3-*f*]chromen-4-one*; **1**). Colorless, fine needles. M.p. 112–114° (PE). $[\alpha]_D^{20} = -30.0$ ($c = 0.10$, MeOH). UV (MeOH): 312 (sh, 3.91), 285 (4.26), 235 (4.36), 220 (sh, 4.51), 208 (4.62). CD (MeOH): 333 (+18,325), 304 (−34,857), 242 (+15,023), 224 (+10,364), 201 (+31,113). IR (KBr): 3473, 2973, 2930, 2857, 1678, 1664, 1600, 1585, 1479, 1444, 1368, 1329, 1268, 1231, 1212, 1156, 1123, 1103, 1059, 1020, 942, 917, 881, 808. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. EI-MS: 478 (31, M^+), 460 (11), 404 (24), 309 (52), 307 (53), 274 (16), 261 (37), 256 (29), 244 (20), 243 (100), 205 (25), 204 (11), 203 (41), 201 (65), 185 (41), 171 (25), 161 (32), 159 (21), 157 (22), 149 (65), 145 (56), 115 (30), 91 (23), 69 (38), 59 (40), 57 (25), 55 (33), 43 (46), 41 (29). HR-ESI-MS: 501.2614 ($[M+Na]^+$, $C_{30}H_{38}NaO_5^+$; calc. 501.2617).

4.2. *Tonkinochromane E* (=*(2*S*)-2,3,9,10-Tetrahydro-2-[3,4-dihydro-8-(3-ethoxy-3-methylbutyl)-2,2-dimethyl-2*H*-chromen-6-yl]-8,8-dimethyl-4*H*,8*H*-pyrano[2,3-*f*]chromen-4-one*; **2**). Colorless oil. $[\alpha]_D^{20} = -47.5$ ($c = 0.30$, MeOH). UV (MeOH): 312 (sh, 3.87), 285 (4.20), 232 (sh, 4.32), 221 (4.39). CD (MeOH): 333 (+15,430), 303 (−29,731), 243 (+15,208), 202 (+33,072). IR (CH_2Cl_2): 2974, 2932, 2866, 1679, 1601, 1583, 1474, 1436, 1370, 1330, 1267, 1208, 1158, 1121, 1100, 1068, 1052, 1008, 942, 882, 822, 734. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. EI-MS: 506 (3, M^+), 460 (26), 404 (13), 256 (14), 243 (35), 205 (9), 204 (4), 203 (17), 201 (22), 185 (18), 161 (18), 159 (13), 157 (10), 149 (49), 145 (25), 115 (16), 91 (14), 87 (47), 69 (19), 59 (100), 57 (17), 55 (29), 43 (40), 41 (31). HR-ESI-MS: 529.2927 ($[M+Na]^+$, $C_{32}H_{42}NaO_5^+$; calc. 529.2930).

4.3. *Tonkinochromane F* (=*(2*E*)-3-[3,4-Dihydro-8-(3-ethoxy-3-methylbutyl)-2,2-dimethyl-2*H*-chromen-6-yl]-1-(3,4-dihydro-5-hydroxy-2,2-dimethyl-2*H*-chromen-6-yl)prop-2-en-1-one*; **3**). Yellow oil. UV (MeOH): 381 (4.64), 245 (sh, 4.14), 209 (4.61). IR (CH_2Cl_2): 3441, 2974, 2928, 2855, 1629, 1588, 1564, 1469, 1432, 1370, 1332, 1272, 1210, 1155, 1109, 1073, 1049, 981, 894, 881, 805, 740. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. EI-MS: 506 (8, M^+), 460 (10), 405 (14), 404 (18), 256 (18), 244 (19), 243 (100), 205 (18), 203 (30), 201 (18), 187 (9), 185 (13), 175 (8), 161 (11), 159 (9), 149 (57), 145 (12), 87 (22), 69 (9), 59 (37), 43 (13). HR-ESI-MS: 507.3105 ($[M+H]^+$, $C_{32}H_{43}O_5^+$; calc. 507.3110).

5. *Qualitative LC-MS Analyses.* 5.1. *Apparatus and Conditions.* LC-MS Analyses were performed on a *Waters Alliance HT 2795-ZQ2000* system equipped with a *2795* separation module, a *2996* photo-diode-array (PDA) detector, and a *ZQ-2000* mass spectrometer. Liquid chromatography was run on a *YMC-Pack ODS-AQ* column (5 μm; 4.6 × 150 mm), eluting with a linear gradient of solvents *B* (=0.2% (*v/v*) AcOH in MeCN) and *A* (=aq. 0.2% (*v/v*) AcOH), starting at 75% *B* to 25% *B* within 15 min, followed by 10 min isocratic elution at a flow rate of 0.8 ml/min. The column temp. was controlled at 45°, the detection wavelength was set at 265 nm, and the injection volume was 18 μl. Ionization was achieved in the pos. mode with an electrospray ion source at a voltage of 3.5 kV, 2.5 kV, 5 V, and 0.5 V for capillary, cone, extractor, and RF lens, resp. The applied temp. was controlled at 110° for the source and at 350° for

the desolvation gas. N_2 was used as a nebulizer and curtain gas, the desolvation gas flow was set to 400 l/h, and the cone gas flow was 50 l/h. Lens and quadrupole parameters were optimized to achieve maximum intensity of the signals. The MassLynx 4.0 software was used for data acquisition and processing.

5.2. *Sample Preparation*. Tonkinochromanes A – F (1 mg each) were mixed together and dissolved in MeOH (5 ml) to give a suitable concentration (0.2 mg/ml) as reference soln. *Sample 1* was prepared as follows: an aliquot (0.4 g) of the EtOH extract of the crude plant previously treated with 1% (*v*/*v*) aq. H_2SO_4 (see above), was dissolved in MeOH (5 ml) to give a concentration of *ca*. 0.5 g/ml. *Sample 2* for comparative analysis was prepared as follows: 5.0 g of the pulverized, intact, crude plant material was macerated with 50 ml of 95% EtOH at r.t. overnight, and then filtered. Part of the filtrate (*ca*. 25 ml) was evaporated to dryness *in vacuo* at 50°, and the resulting residue was dissolved in MeOH (5 ml) to give a concentration of 0.5 g/ml. All of the above solns. were passed through a 0.2-μm membrane filter before analysis and subjected to LC-MS analysis under the above-mentioned exper. conditions.

5.3. *LC-MS Data. Sample 1* (see *Fig. 3*): t_R 8.5 min (*peak 1*): *m*/*z* 479 (27, $[M+H]^+$), 461 (100, $[M-OH]^+$); t_R 15.1 min (peak *2*): *m*/*z* 507 (6, $[M+H]^+$), 461 (100, $[M-OCH_2CH_3]^+$); t_R 20.2 min (peak *3*): *m*/*z* 507 (38, $[M+H]^+$), 461 (100, $[M-OCH_2CH_3]^+$); t_R 15.4 min (peak *4*): *m*/*z* 461 (100, $[M+H]^+$); t_R 11.4 min (peak *5*): *m*/*z* 461 (100, $[M+H]^+$); t_R 19.5 min (peak *6*): *m*/*z* 459 (100, $[M+H]^+$)). *Peaks 1–6* represent tonkinochromanes D, E, F, A, B, and C, resp. All of the above peaks were identical to those of the corresponding reference flavonoids. No tonkinochromanes were detected by analysis of *Sample 2* (lacking acid pretreatment).

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