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ORIGINAL PAPER

Evaluation of the chemical quality of *Sekkoku* (石斛) in current Japanese commercial crude drugs: constituents of *Flickingeria xantholeuca* (Rchb. f.) A.D. Hawkes

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Abstract In our investigation, most *Shihu* (石斛; Japanese name, *Sekkoku*) in current Japanese commercial crude drugs were from *Flickingeria xantholeuca* (Orchidaceae). As the index compounds, three new *ent*-pimarane-type diterpenes, flickinxthanthosides A–C (**1**–**3**), one known analogue (**7**), and three new *ent*-kaurane-type diterpenes, flickinxanthosides D (**4**) and E (**5**) and flickinxanthol A (**6**) were isolated from the stem of *F. xantholeuca*. The structures of the new compounds were elucidated on the basis of spectroscopic analyses and chemical methods. We attempted to detect these index compounds from the MeOH extracts of other *Dendrobium* or *Flickingeria* plants using TLC and LC/MS.

Keywords *ent*-Pimarane-type diterpene · *ent*-Kaurane-type diterpene · Glycoside · *Flickingeria xantholeuca* · Orchidaceae · TLC · LC/MS

Introduction

The Chinese herbal medicine *Shihu* (石斛) is prepared from the stem of several *Dendrobium* plants (Orchidaceae), such as *Dendrobium nobile*, *Dendrobium fimbriatum*, and *Dendrobium chrysanthum*. *Dendrobium officinale* Kimura et Migo (=*D. catenatum* Lindl), called *Tiepishihu* (鉄皮石斛) in Chinese, is also a species in the large *Dendrobium* genus. The various *Dendrobium* species are indicated for improving immune system function and as antioxidant, antifatigue, antihyperglycemia, and anticancer agent, etc., in the Chinese Pharmacopoeia [1–3]. *Tiepishihu* is a highly regarded medicinal plant in Chinese culture, while *Dendrobium moniliforme*, *Dendrobium tosaense* Makino (=*D. catenatum* Lindl), and *Dendrobium okinawense* are distributed widely in Japan. The Kampo formulation Kanroin (甘露飲) is included in the guidelines (revised edition) for the approval standards for over-the-counter Kampo products. However, *Shihu* (in Japanese *Sekkoku*) is not recorded in the seventeenth edition of the Japanese Pharmacopoeia. Our investigations showed that although *Flickingeria xantholeuca* (Rchb. f.) A.D. Hawkes [=*Ephemerantha lonchophylla* (Hook. f.) P.F. Hunt et Summerh.] was previously the source of *Shihu* in PR China [4], most *Sekkoku* in current Japanese commercial crude drugs are from *F. xantholeuca* (戟葉金石斛).

F. xantholeuca is a source of secondary metabolites, such as phenanthrenes, 9,10-dihydrophenanthrenes, *cis*-clerodane-type diterpene lactones, and *ent*-pimarane-type diterpenes [4, 5]. With the aim of developing chemical quality evaluation criteria for *Sekkoku*, we investigated the index compounds of this herb and isolated three new *ent*-pimarane-type diterpenes, flickinxthanthosides A–C (**1**–**3**), one known analogue (**7**) [4, 6], and three new *ent*-kaurane-type diterpenes, flickinxanthosides D (**4**) and E (**5**) and flickinxanthol A (**6**) (Fig. 1).

This paper reports the isolation and structural elucidation of the six new natural products as well as our attempts to detect the index compounds from the MeOH extracts of other *Dendrobium* or *Flickingeria* plants using thin-layer

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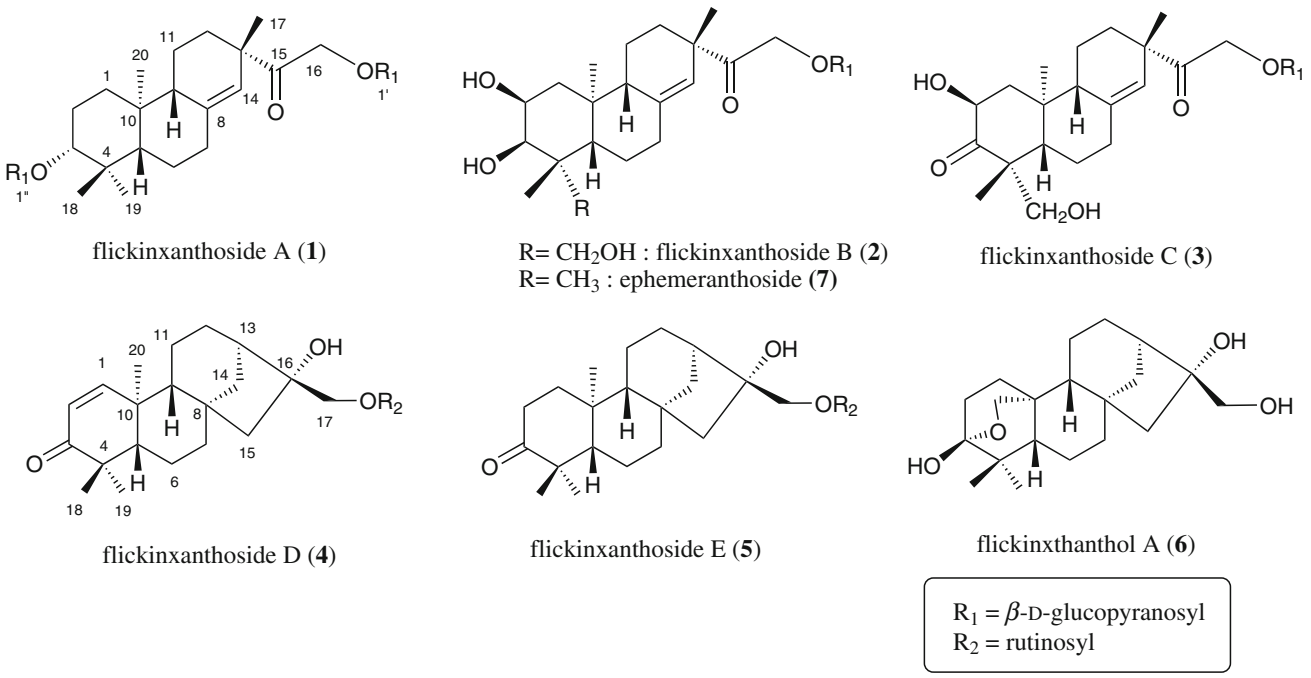

Fig. 1 Chemical structures of compounds **1**–**7**

chromatography (TLC) and ultra-high-performance liquid chromatography/mass spectrometry (LC/MS).

Results and discussion

Research on current Japanese commercial crude drugs

We investigated several types of *Sekkoku* produced by different Japanese companies. Because they were all cut crude drugs, it was not possible to identify the original plant sources by simple observation. The herb was imported to Japan from PR China and Vietnam as botanical specimens before the signing of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The plants were then grown in the medicinal plant garden of Osaka University of Pharmaceutical Sciences. We investigated the botanical specimens and identified them as *F. xantholeuca* (Rchb. f.) A.D. Hawkes [=*Ephemerantha lonchophylla* (Hook. f.) P.F. Hunt et Summerh.] (戟葉金石斛) [7], as shown in Fig. 2. During a field survey in Myanmar, we obtained *F. xantholeuca* specimens from the Department of Agriculture, Ministry of Agriculture and Irrigation.

F. xantholeuca was previously the source of *Shihu* in PR China, and most *Sekkoku* in current Japanese commercial crude drugs is from *F. xantholeuca* (戟葉金石斛). One reason for this is because orchidaceous plants are protected by CITES, and the *Sekkoku* sources of the current Japanese commercial crude drugs were imported before CITES enforcement. In addition, *Dendrobium* plants have never been cultivated as medicinal herbs, although three *Dendrobium* genera [*D. moniliforme, D. tosaense* Makino (=*D. catenatum* Lindl), and *D. okinawense*] grow naturally in Japan.

Structures of index compounds

The aerial parts of this plant were extracted with hot methanol by reflux, and the extract was chromatographed on an NH silica gel column and subjected to preparative HPLC to provide the purified diterpenes **1**–**7** as described in the Experimental section.

Flickinxthanthoside A (**1**) was obtained as a colorless powder, α_D^{25} −62.1 (c = 0.32, MeOH), showing a reddish purple spot on TLC when sprayed with a 20 % sulfuric acid reagent, followed by heating on a hotplate. The molecular formula was determined to be $C_{32}H_{52}O_{13}$ on the basis of HR-FABMS (*m/z*: 667.3309, $[M+Na]^+$, calcd. 667.3305). The IR spectrum showed a strong OH band and C = O band at 3392 and 1718 cm^{-1}, respectively.

The ^1H NMR spectrum of **1** showed the presence of four tertiary methyl groups [δ 0.56, 0.85, 1.04, 1.18 (each 3*H*, s)], one olefin group [δ 5.47 (1*H*, s)], one oxygenated methine group [δ 3.52 (1*H*, dd, J = 12.0, 4.2 Hz)], one oxygenated methylene group [δ 4.94 (1*H*, d, J = 18.0 Hz), 5.13 (1*H*, d, J = 18.0 Hz)], and two β-form glucopyranosyl moieties [δ 5.00 (1*H*, d, J = 7.8 Hz, H-1′), δ 4.13 (1*H*, br. t, J = 8.4 Hz, H-2′), δ 4.29 (1*H*, dd, J = 9.0, 9.0 Hz, H-3′), δ

Fig. 2 *Sekkoku* as the current Japanese commercial crude drug and its original plants. **a** F. xantholeuca growing at the Department of Agriculture, Ministry of Agriculture and Irrigation, in Myanmar. **b** F. xantholeuca growing at Osaka University of Pharmaceutical Sciences.**c** Current Japanese commercial crude drug (whole crude drug).**d** Current Japanese commercial crude drug (cut crude drug)

4.22 (1*H*, dd, $J = 9.0$, 9.0 Hz, H-4′), δ 3.90 (1*H*, m, H-5′), δ 4.34 (1*H*, dd, $J = 12.0$, 6.0 Hz, H-6′), δ 4.49 (1*H*, dd, $J = 12.0$, 1.8 Hz, H-6′), and δ 4.88 (1*H*, d, $J = 7.8$ Hz, H-1″), δ 4.00 (1*H*, br. t, $J = 8.4$ Hz, H-2″), δ 4.28 (1*H*, dd, $J = 9.0$, 9.0 Hz, H-3″), δ 4.19 (1*H*, dd, $J = 9.0$, 9.0 Hz, H-4″), δ 3.98 (1*H*, m, H-5″), δ 4.31 (1*H*, dd, $J = 12.0$, 6.0 Hz, H-6″), δ 4.57 (1*H*, dd, $J = 12.0$, 1.8 Hz, H-6″)] (Table 1). In the ^{13}C-NMR and DEPT spectra, a total of 32 carbons, composed of four methyls, seven methylenes, three methines, three quaternary carbons, one endocyclic double bond, and one carbonyl carbon, including 12 signals assignable to two glucose moieties (δ 104.0, 74.8, 77.8, 71.0, 78.3, 62.1 and δ 102.0, 74.8, 78.1, 71.6, 78.0, 62.7), were observed (Table 2). These data were similar to those of ephemeranthoside (**7**) [4, 6], which was previously isolated from the same plant, except for the presence of two glucose moieties and a methylene signal (δ 1.51 m, δ 1.97) instead of the C-2 methine proton [δ 4.21 (1*H*, ddd, $J = 12.0$, 4.0, 2.0 Hz)] adjacent to an oxygen atom of **7**. Acid hydrolysis with 5 M HCl–CH_3OH (1:3) of **1** gave D-glucose (α_D^{25} +57.8). Its connectivity to aglycon in **1** was confirmed by the long-range correlation of the signals of H-1′ and H-1″ with the signal of C-16 (δ 71.4) and C-3 (δ 84.8) in the HMBC experiment (Fig. 3). The relative configuration of **1** was determined through the NOESY correlations between H-18/H-3, H-3/H-5, H-5/H-9, H-19/H-20 and H-20/H-16 (Fig. 4). On the basis of this evidence, the structure of **1** was formulated as 3-*O*-β-D-glucopyranosyl-3β, 16-dihydroxy-15-keto-*ent*-pimara-8(14)-ene 16-*O*-β-D-glucopyranoside, as shown in Fig. 1.

Flickinxthanthoside B (**2**) was obtained as a colorless powder, α_D^{25} −37.3 ($c = 0.87$, MeOH), showing a reddish purple spot on TLC when sprayed with a 20 % sulfuric acid reagent, followed by heating on a hotplate. The molecular formula was determined to be $C_{26}H_{42}O_{10}$ on the basis of HR-FABMS (*m/z*: 515.2863, $[M+H]^+$, calcd. 515.2857). The IR spectrum showed a strong OH band and C = O band at 3400 and 1714 cm^{-1}, respectively.

The 1*H* NMR spectrum was similar to that of **7**, except for the presence of a methylene signal [δ 3.79 (1*H*, d, $J = 11.1$ Hz), δ 4.13 (1*H*, d, $J = 11.1$ Hz)] adjacent to an oxygen atom instead of the C-19 methyl proton [δ 0.88 (3*H*, s)] of **7**. Acid hydrolysis of **2** with 5 M HCl–CH_3OH (1:3) gave D-glucose (α_D^{25} +45.0). Its connectivity to the aglycon in **2** was confirmed by the long-range correlation of the signals of H-1′ with the signal of C-16 (δ 71.6) in the HMBC experiment. The ^{1}H and ^{13}C NMR data were assigned based on the ^{1}H–^{1}H COSY, HSQC, HMBC, and DEPT spectra (Fig. 3, Tables 1, 2). The relative configuration of **2** was determined through the NOESY correlations between H-2/H-20, H-20/H-19, H-20/H-16, H-18/H-5 and H-5/H-9 (Fig. 4). Thus, the structure of **2** was formulated as 2α, 3α, 16, 19-tetrahydroxy-15-keto-*ent*-pimara-8(14)-ene 16-*O*-β-D-glucopyranoside, as shown in Fig. 1.

Flickinxthanthoside C (**3**) was obtained as a colorless powder, α_D^{25} −34.6 ($c = 0.46$, MeOH), showing a brownish

Table 1 ^{1}H NMR spectral data for **1**–**6** (δ ppm, in pyridine-d_5)

	1	**2**	**3**		**4**	**5**	**6**
1	0.89 ddd (13.8, 13.8, 3.0), 1.40 m	1.80 m, 2.06[a]	1.53 dd (12.0, 12.0), 2.36 dd (12.0, 5.5)	1	7.10 d (10.2)	1.20 m, 1.84 ddd (13.2, 6.6, 6.6)	1.10[a], 2.22 td (12.5, 5.5)
2	1.51 m, 1.97[a]	4.48[b]	4.94 dd (12.0, 5.5)	2	5.96 d (10.2)	2.47[a]	2.10[b], 2.37 td (12.5, 5.5)
3	3.52 dd (12.0, 4.2)	4.63 d (2.3)		3			
4				4			
5	0.94 dd (12.6, 2.4)	1.99 dd (13.2, 2.3)	1.72 m	5	1.50 dd (11.4, 2.4)	1.32[b]	1.26 m
6	1.22 m, 1.42 m	1.42 m, 1.72 m	1.30 m, 1.48[a]	6	1.36 m	1.32[b]	1.50[c], 1.64[d]
7	1.97,[a] 2.23 ddd (11.4, 4.2, 2.4)	2.06,[a] 2.29 dd (14.4, 2.6)	1.00 m, 2.38[b]	7	1.41 m, 1.64 m	1.41 m, 1.59[c]	1.42 td (13.0, 3.5), 1.63[c]
8				8			
9	1.55 m	1.91 m	1.60[c]	9	1.13 br.d (7.2)	0.98 d (8.4)	1.12[a]
10				10			
11	1.17 m, 1.47 m	1.33 m, 1.58 m	1.48,[a] 1.60[c]	11	α: 1.63 m, β: 1.74 m	1.50 m	1.54[c], 1.63[d]
12	1.00 ddd (13.2, 13.2, 3.6), 2.38 br.d (12.6)	0.98 ddd (16.2, 13.2, 3.0), 2.40 d (12.1)	2.01 m, 2.27 m	12	1.46 m, 2.05 m	1.47 m, 1.96[d]	1.18 m, 1.82 m
13				13	2.47 br.d (3.6)	2.47[a]	2.38 m
14	5.47 s	5.47 s	5.52 br.s	14	α: 1.77 m, β: 1.99 m	α: 1.81 d (13.8), β: 1.96[d]	α: 1.52[c], β: 2.08[b]
15				15	α: 1.67 d (15.0), β: 1.80 d (15.0)	α: 1.59[c], β: 1.78 d (14.4)	α: 1.68 br.d (14.0), β: 1.81 br.d (14.0)
16	4.94 d (18.0), 5.13 d (18.0)	4.99 d (17.9), 5.08 d (17.9)	5.00 d (17.7), 5.11 d (17.7)	16			
17	1.04 s	1.02 s	1.05 s	17	a: 4.00 d (10.2), b: 4.52 d (10.2)	a: 3.99 d (10.2), b: 4.51 d (10.2)	a: 4.04 d (10.5), b: 4.12 d (10.5)
18	1.18 s	1.64 s	1.54 s	18	1.16 s	1.09 s	1.24 s
19	0.85 s	3.79 d (11.1), 4.13 d (11.1)	3.78 d (11.0), 4.42 d (11.0)	19	1.10 s	1.01 s	1.32 s
20	0.56 s	0.80 s	1.07 s	20	1.08 s	0.92 s	3.93 dd (9.0, 1.8), 4.57 dd (9.0, 2.5)
Glc-1′	5.00 d (7.8)	5.04 d (7.8)	5.09 d (7.5)	Glc-1′	5.06 d (7.8)	5.00 d (7.8)	
2′	4.13 br.t (8.4)	4.15 br.t (8.4)	4.17 dd (9.0, 7.5)	2′	4.12 dd (9.0, 7.8)	4.12[e]	
3′	4.29 dd (9.0, 9.0)	4.28[c]	4.31 dd (9.0, 9.0)	3′	4.26 dd (9.0, 9.0)	4.25 dd (9.0, 9.0)	
4′	4.22 dd (9.0, 9.0)	4.28[c]	4.26 dd (9.0, 9.0)	4′	4.06 dd (9.0, 9.0)	4.06 dd (9.0, 9.0)	
5′	3.90 m	3.94 m	3.97 m	5′	4.14 m	4.13[e]	
6′	4.34 dd (12.0, 6.0), 4.49 dd (12.0, 1.8)	4.48,[b] 4.54 dd (12.0, 2.4)	4.38 dd (12.0, 5.5), 4.56 dd (12.0, 2.0)	6′	4.23 dd (11.4, 6.6), 4.74 dd (11.4, 1.8)	4.21 dd (10.8, 6.0), 4.71 dd (10.8, 1.2)	
Glc-1″	4.88 d (7.8)			Rha-1′′	5.56 d (1.2)	5.54 d (1.2)	
2″	4.00 br.t (8.4)			2″	4.66 br.s	4.64 br.s	
3″	4.28 dd (9.0. 9.0)			3″	4.59 dd (9.0, 3.0)	4.57 dd (9.0, 3.0)	
4″	4.19 dd (9.0, 9.0)			4″	4.30 dd (9.0, 9.0)	4.29 dd (9.0, 9.0)	
5″	3.98 m			5″	4.42 m	4.40 m	
6″	4.31 dd (12.0, 6.0), 4.57 dd (12.0, 1.8)			6″	1.68 d (6.6)	1.66 d (6.0)	

Values in parentheses are coupling constants in Hertz

[a–e] Overlapping signals

purple spot on TLC when sprayed with a 20 % sulfuric acid reagent, followed by heating on a hotplate. The molecular formula was determined to be $C_{26}H_{40}O_{10}$ on the basis of HR-FABMS (*m/z*: 535.2527, $[M+Na]^+$, calcd. 535.2519). The IR spectrum showed a strong OH band and C = O band at 3399 and 1715 cm^{-1}, respectively.

The ^{1}H NMR spectrum was similar to that of **2**, except for the disappearance of the C-3 oxymethine signal [δ 4.63

Table 2 ^{13}C NMR spectral data for **1**–**6** (δ ppm, in pyridine-d_5)

	1	2	3		4	5	6
1	36.2	40.8	48.2	1	160.6	39.2	35.9
2	23.6	66.4	70.9	2	125.6	34.2	30.6
3	84.8	74.1	214.7	3	204.6	216.9	98.0
4	38.2	39.8	38.9	4	44.9	47.1	40.8
5	54.3	48.8	50.4	5	53.5	54.2	50.1
6	22.1	22.4	20.9[a]	6	21.2	21.9	22.2
7	35.5	36.5	32.8	7	41.5	41.3	40.0
8	141.6	142.0	140.6	8	44.8	44.5	44.0
9	50.3	51.1	57.4	9	50.1	55.5	50.7
10	38.3	45.1	55.2	10	41.6	38.6	37.2
11	20.1	20.8	23.2	11	18.8[a]	19.0	19.2
12	32.7	33.0	35.7	12	26.4	26.5	26.6
13	47.4	47.7	47.7	13	46.1	46.2	45.2
14	124.1	124.7	125.7	14	38.0	37.2	38.2
15	210.1	210.6	210.3	15	53.0	53.0	52.6
16	71.4	71.6	71.6	16	80.8	80.8	82.0
17	26.8	27.1	27.0	17	75.3	75.2	66.3
18	28.6	23.8	20.9[a]	18	28.4	27.3	27.5
19	16.9	65.1	65.7	19	21.6	21.1	19.4
20	14.1	16.5	16.2	20	21.4	17.8	68.4
Glc-1′	104.0	104.4	104.4	Glc-1′	106.1	106.1	
2′	74.8	75.3	75.3	2′	75.4	75.4	
3′	77.8	78.4	78.4	3′	78.7	78.6	
4′	71.0	71.5	71.5	4′	72.0	71.9	
5′	78.3	78.8	78.8	5′	77.2	77.1	
6′	62.1	62.6	62.7	6′	68.7	68.5	
Glc-1″	102.0			Rham-1″	102.8	102.7	
2″	74.8			2″	72.4	72.3	
3″	78.1			3″	72.9	72.8	
4″	71.6			4″	74.1	74.1	
5″	78.0			5″	69.9	69.8	
6″	62.7			6″	18.8[a]	18.8	

Values in parentheses are coupling constants in Hz

(1*H*, d, J = 2.3 Hz)] in **2**. In the ^{13}C NMR spectrum, one carbonyl carbon signal (δ 214.7) was observed instead of the oxymethine carbon signal (δ 74.1) of **2**. The ^{1}H and ^{13}C NMR data were assigned based on the ^{1}H–^{1}H COSY, HSQC, HMBC, and DEPT spectra (Fig. 3, Tables 1, 2). The relative configuration of **3** was determined through the NOESY correlations between H-2/H-20, H-20/H-19, H-20/H-16, H-18/H-5 and H-5/H-9 (Fig. 4). Acid hydrolysis of **3** with 5 M HCl–CH_3OH (1:3) gave D-glucose (α_D^{25} +51.4°). Thus, the structure of **3** was formulated as 2α,16,19-trihydroxy-3,15-diketo-*ent*-pimara-8(14)-ene 16-*O*-β-D-glucopyranoside, as shown in Fig. 1.

Flickinxthanthoside D (**4**) was obtained as a colorless powder, α_D^{25} −66.3 (c = 0.28, MeOH), showing a brownish spot on TLC when sprayed with a 20 % sulfuric acid reagent, followed by heating on a hotplate. The molecular formula was determined to be $C_{32}H_{50}O_{12}$ on the basis of HR-FABMS (*m/z*: 627.3386, $[M+H]^+$, calcd. 627.3381). The IR spectrum showed a strong OH band and an α,β-unsaturated C = O band at 3434 and 1669 cm^{-1}, respectively.

The ^{1}H NMR spectrum of **4** showed the presence of three tertiary methyl groups [δ 1.16, 1.10, 1.08 (each 3*H*, s)], one olefin group [δ 5.96 (1*H*, d, J = 10.2), δ 7.10 (1*H*, d, J = 10.2)], one oxygenated methylene group [δ 4.00 (1*H*, d, J = 10.2 Hz), 4.52 (1*H*, d, J = 10.2 Hz)], and one rutinose moiety [δ 5.06 (1*H*, d, J = 7.8 Hz, H-1′), δ 4.12 (1*H*, dd, J = 9.0, 7.8 Hz, H-2′), δ 4.26 (1*H*, dd, J = 9.0, 9.0 Hz, H-3′), δ 4.06 (1*H*, dd, J = 9.0, 9.0 Hz, H-4′), δ 4.14 (1*H*, m, H-5′), δ 4.23 (1*H*, dd, J = 11.4, 6.6 Hz, H-6′), δ 4.74 (1*H*, dd, J = 11.4, 1.8 Hz, H-6′), and δ 5.56 (1*H*, d, J = 1.2 Hz, H-1″), δ 4.66 (1*H*, br. br.s, H-2″), δ 4.59 (1*H*, dd, J = 9.0, 3.0 Hz, H-3″), δ 4.30 (1*H*, dd, J = 9.0, 9.0 Hz, H-4″), δ 4.42 (1*H*, m, H-5″), δ 1.68 (3*H*, d, J = 6.6 Hz, H-6″)] (Table 1). In the ^{13}C NMR and DEPT spectra, a total of 32 carbons, composed of three methyls, seven methylenes, three methines, four quaternary carbons, one endocyclic double bond, and one carbonyl carbon, including 12 signals assignable to one rutinose moiety (δ 106.1, 75.4, 78.7, 72.0, 77.2, 68.7 and δ 102.8, 72.4, 72.9, 74.1, 69.9, 18.8), were observed (Table 2).

Each partial structure was obtained by the analyses of ^{1}H–^{1}H COSY cross-peaks and they were connected on the basis of the HMBC spectrum to establish the planar structure (Fig. 3). The relative configuration of **4** was determined through the NOESY correlations between H-18/H-5, H-5/H-9, H-9/H-15β, H-15β/H-11β, H-11β/H-17b, H-15β/H-17a, H-19/H-20 and H-20/H-14α, as shown in Fig. 4. Acid hydrolysis of **4** with 5 M HCl–CH_3OH (1:3) gave D-glucose (α_D^{25} +47.8°) and L-rhamnose (α_D^{25} +10.4°). The absolute configuration of **4** was determined by its circular dichroism (CD) spectrum (Fig. 5). The CD curve showed a negative Cotton effect at 238 nm ($\Delta\varepsilon$ –5.82) from the $\pi \rightarrow \pi^*$ transition and a positive Cotton effect at 342.4 nm ($\Delta\varepsilon$ +1.55) from the $n \rightarrow \pi^*$ transition. The positive Cotton effect allowed the assignment of the configuration (5*S*, 8*S*, 9*R*, 10*S*, 13*R*, 16*R*) for **4** according to the reversed octant rule of α,β-unsaturated ketone [8, 9]. On the basis of this evidence, the structure of **4** was formulated as (16*R*)-16,17-dihydroxy-3-keto-*ent*-kaura 1(2)-ene 17-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside, as shown in Fig. 1.

Flickinxthanthoside E (**5**) was obtained as a colorless powder, α_D^{25} −72.4 (c = 0.40, MeOH), showing a brownish spot on TLC when sprayed with a 20 % sulfuric acid reagent, followed by heating on a hotplate. The molecular formula was determined to be $C_{32}H_{52}O_{12}$ on the basis of HR-FABMS (*m/z*: 651.3343, $[M+Na]^+$, calcd. 651.3356).

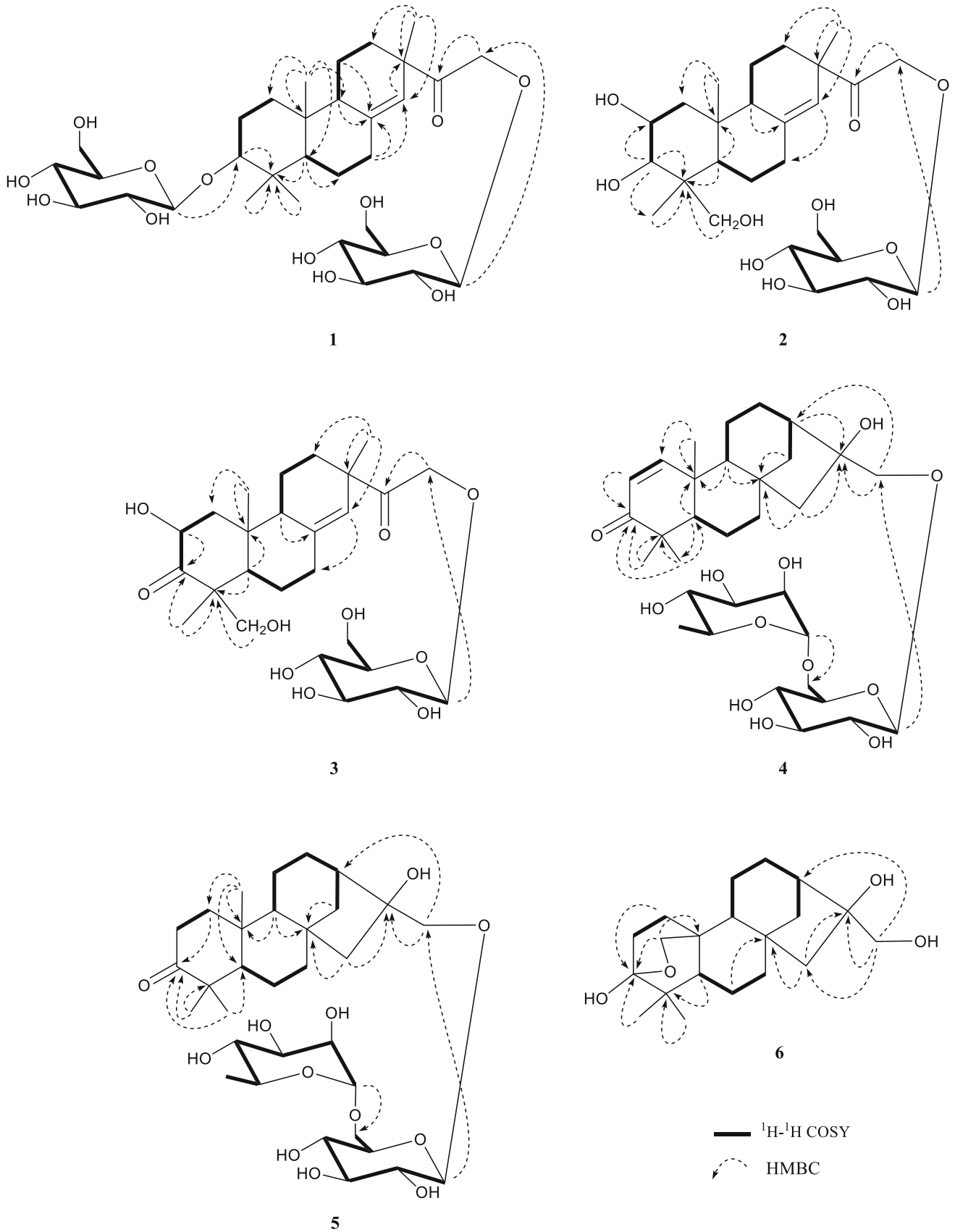

Fig. 3 Selected HMBC and ^{1}H–^{1}H COSY correlations in **1**–**6**

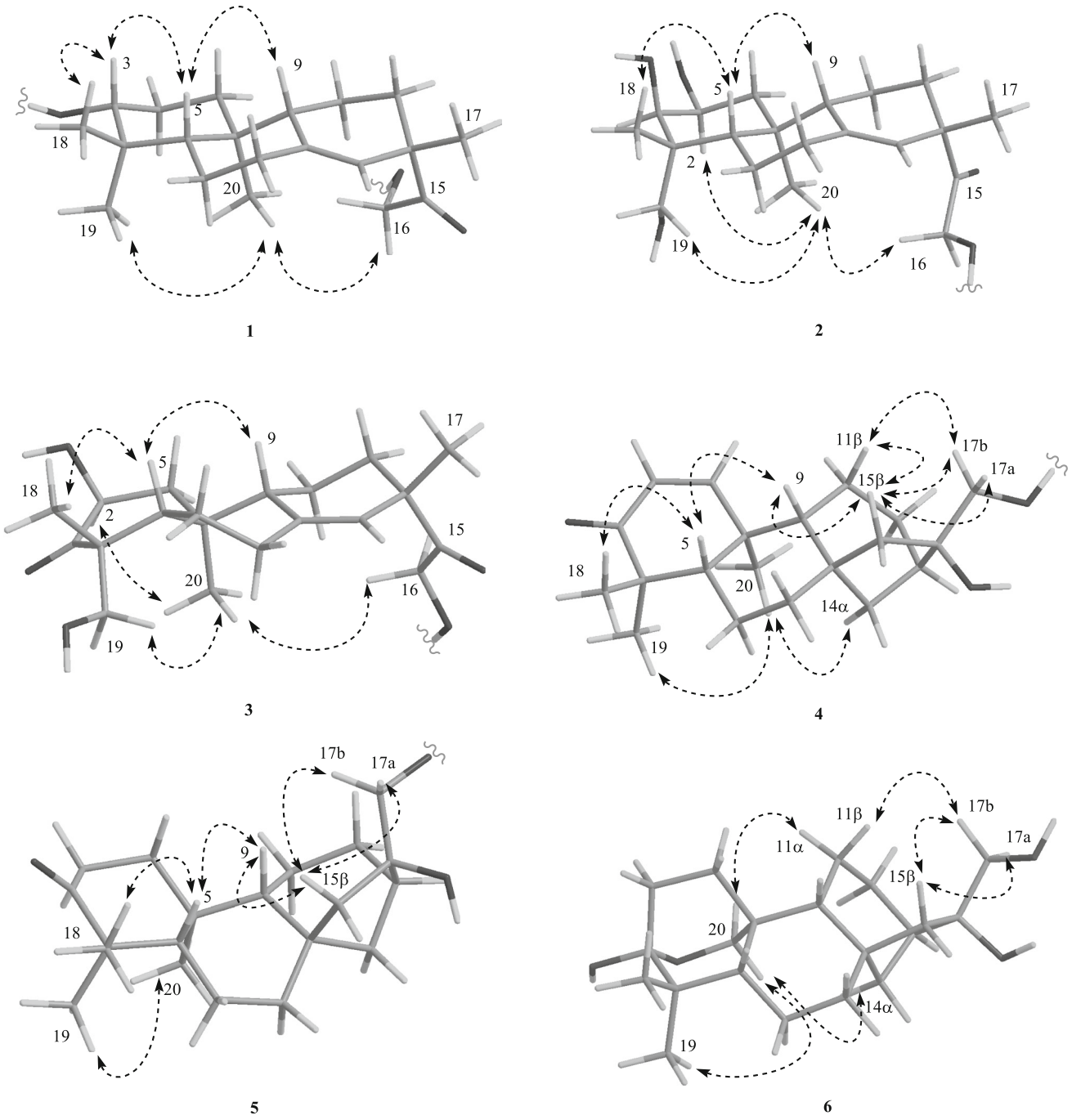

Fig. 4 Selected NOESY correlations in **1**–**6**

The IR spectrum showed a strong OH band and C = O band at 3399 and 1699 cm^{-1}, respectively.

The ^{1}H NMR spectrum was similar to that of **4**, except for the presence of two methylene signals [δ 1.20 (1*H*, m), δ 1.84 (1*H*, ddd, $J = 13.2, 6.6, 6.6$ Hz)] instead of the C-1 and C-2 olefin protons [δ 5.96 (1*H*, d, $J = 10.2$ Hz), δ 7.10 (1*H*, d, $J = 10.2$ Hz)] of **4**. Each partial structure was obtained by the analyses of ^{1}H–^{1}H COSY cross-peaks, and they were connected on the basis of the HMBC spectrum to establish the planar structure (Fig. 3). The ^{1}H and ^{13}C NMR data were assigned based on the ^{1}H–^{1}H COSY, HSQC, HMBC, and DEPT spectra (Tables 1, 2). The relative configuration was determined through the NOESY correlations between H-18/H-5, H-5/H-9, H-9/H-15β, H-15β/H-17a, and H-15β/H-17b as well as **4** (Fig. 4). Thus, the structure of **5** was formulated as 16α,17-dihydroxy-3-keto-*ent*-kaurane 17-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside, as shown in Fig. 1.

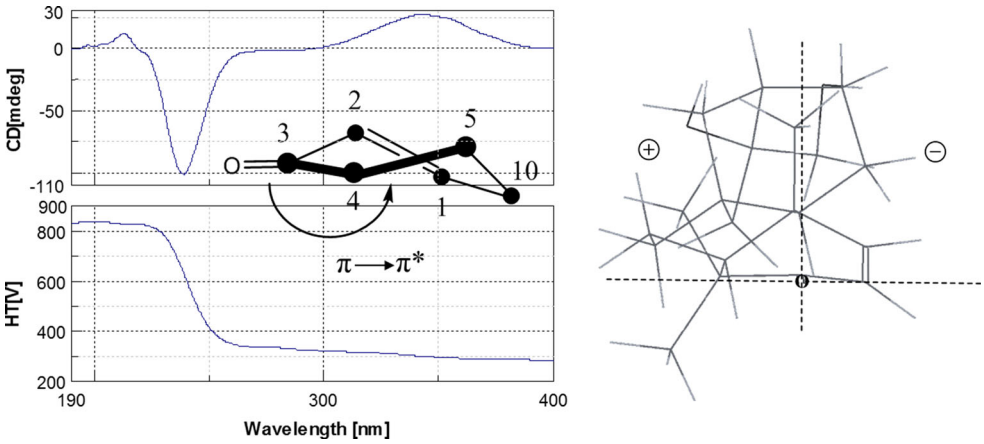

Fig. 5 Circular dichroism (CD) spectrum and octant projection of **4**

Flickinxthanthol A (**6**) was obtained as a colorless powder, α_D^{25} −26.4 ($c = 0.41$, MeOH), showing a brownish spot on TLC when sprayed with a 20 % sulfuric acid reagent, followed by heating on a hotplate. The molecular formula was determined to be $C_{20}H_{32}O_4$ on the basis of HR-FABMS (*m/z*: 337.2380, $[M+H]^+$, calcd. 337.2378). The IR spectrum showed a strong OH band at 3411 cm^{-1}.

The ^1H NMR spectrum showed the presence of one oxymethylene signal [δ 3.93 (1*H*, dd, $J = 9.0$, 1.8 Hz), δ 4.57 (1*H*, dd, $J = 9.0$, 1.8 Hz)] instead of the C-20 methyl signal, and the signals from rutinose disappeared. Each partial structure was obtained by analyses of the ^1H-^1H COSY cross-peaks, and they were connected on the basis of the HMBC spectrum to establish the planar structure (Fig. 3). The ^1H and ^{13}C NMR signals were assigned based on the results of HSQC and DEPT experiments, as shown in Tables 1 and 2. The relative configuration was determined through the NOESY correlations between H-20/H-11α, H-20/H-14α, H-20/H-19, H-11β/H-17b, H-15β/H-17b and H-15β/H-17a as shown in Fig. 4. Thus, the structure of **6** was formulated as 3β,16α,17-trihydroxy-3,20-epoxy-*ent*-kaurane, as shown in Fig. 1.

Analyses of MeOH extracts from other *Dendrobium* or *Flickingeria* plants

The total ion chromatogram (TIC) of LC/MS for a MeOH extract of *F. xantholeuca* was obtained and compared with those of *D. moniliforme* and *D. tosaense*. The results indicated that the TIC of *F. xantholeuca* was detected as main index compounds **1**, **2**, **3**, **4**, **5** and **7**, which were not detected from other *Dendrobium* and *Flickingeria* plants (Fig. 6). Thus, the characteristic TIC had three major peaks of index compounds. Moreover, all of these compounds were detected by TLC with the mobile phase EtOAc: MeOH:H_2O = 14:5:4 (Fig. 7). The chemical constituents of *F. xantholeuca*, sold commercially as *Shihu* in Japan, were markedly different from those of *Dendrobium* plants and other *Flickingeria* plants. The decision on which original plant source of *Shihu* to use in the future should be based on this evidence. We are currently researching the biological activities of these index compounds.

Experimental

General

The instruments used in this study were: a JASCO (Osaka, Japan) digital polarimeter (for specific rotation, measured at 25°C); JASCO J-820 spectrometer (for CD measured at 25°C); JASCO FT/IR-680Plus spectrometer (for IR spectra); a JEOL-MS700 V mass spectrometer (for MS spectra); a Agilent VNMRS-600 spectrometer (Santa Clara, CA), operating at 600 MHz for protons and 150 MHz for carbons (for NMR spectra measured in CD_3OD, on the δ scale using tetramethylsilane as an internal standard); a Shimadzu HPLC system (Kyoto, Japan); and a Waters Acquity TQD LC/MS/MS system (Milford, MA). Column chromatography was performed using silica gel NH or DNH (Fuji Silycia Chemical, Aichi, Japan). Silica gel 60F_{254}-precoated TLC plates were purchased from Merck (Darmstadt, Germany), and detection was carried out by spraying with a 20 % sulfuric acid reagent.

Plant material

Air-dried stems of *F. xantholeuca* (Orchidaceae) were kindly provided by Mae Chu Co. (Nara, Japan). This herb was collected in Guangxi, PR China, and imported to Japan with other botanical specimens before CITES came into effect. The authors investigated the botanical specimens and identified them as *F. xantholeuca*. This plant was propagated in the medicinal plant garden of Osaka University of Pharmaceutical Sciences. *D. moniliforme*, *D.*

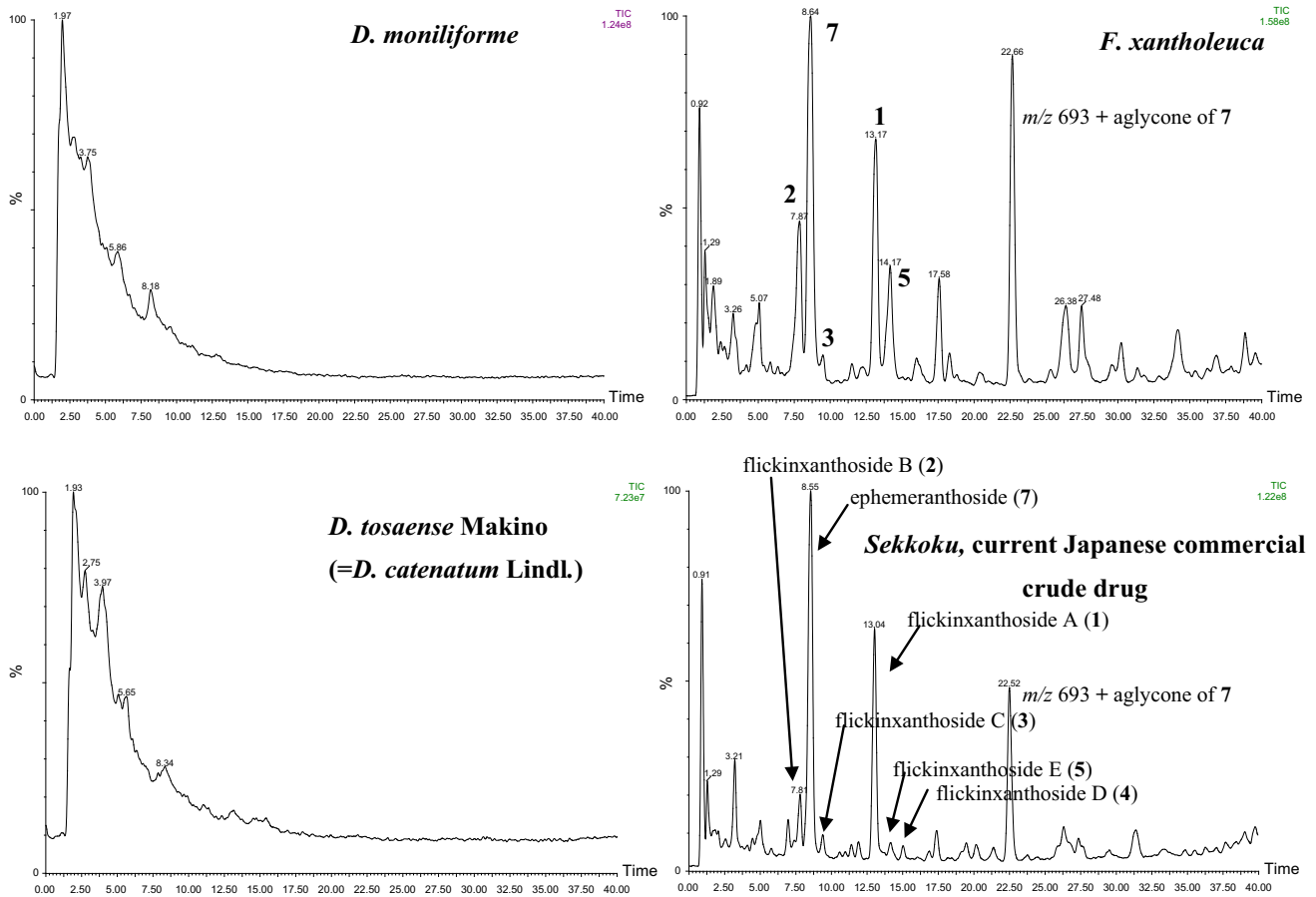

Fig. 6 Total ion chromatogram (TIC) of MeOH extracts of *Sekkoku*, *Dendrobium moniliforme*, *Dendrobium tosaense*, and *Flickingeria xantholeuca* by LC/MS

tosaense Makino (=*D. catenatum* Lindl), *D. nobile*, *F. comata*, and *F. fimbriata* were also grown in the same medicinal plant garden.

Extraction and isolation

The dried stems (400 g) were chopped into small pieces and refluxed with methanol (3 L × 3). The combined methanol extracts were concentrated to dryness in vacuo. The residue (59.2 g) was subjected to column chromatography on NH-silica gel (250 g) eluted successively with a CH_2Cl_2–MeOH solvent system with increasing polarity (10:1 → 1:1) to afford 31 fractions. Fractions 18–30 were rechromatographed on a preparative HPLC column [Cosmosil $5C_{18}$-MS-II or $5C_{18}$-AR-II or 5PE or Cholester (Nacalai Tesque, Kyoto, Japan) 10 × 250 mm; mobile phase, 50 % CH_3OH; flow rate, 1.2 mL/min; detection, RI; column temperature, room temperature] to give **1** (59.6 mg), **2** (16.8 mg), **3** (9.6 mg), **4** (17.1 mg), **5** (4.6 mg), **6** (8.0 mg), and **7** (100.0 mg), respectively.

LC/MS analysis

Powder (1 g) of the dried aerial part of *Dendrobium* or *Flickingeria* plants was extracted with CH_3OH (10 mL) by reflux for 15 min. The filtrates were subjected to LC/MS analysis [column, Cosmosil 2.5C18 (2.0 i.d. × 100 mm); solvent A, 5 % CH_3CN (0.1 % formic acid); solvent B, 60 % CH_3CN (0.1 % formic acid)]. A mobile-phase gradient was used with the percentage of B in A varied as follows: initial concentration, 20 % B; 40 min, 40 % B; 41 min, 20 % B; 45 min, 20 % B; flow rate, 0.2 ml/min; column temperature, 40 °C; MS, positive electrospray ionization (ESI); cone voltage, 40 V; injection, 5 µl (autosampler).

Flickinxthanthoside A (1)

A colorless powder, α_D^{25} −62.1 (c = 0.32, MeOH), HR-positive FAB-MS *m/z*: 667.3309, $[M+Na]^+$, calcd. 667.3305 ($C_{32}H_{52}O_{13}Na$), IR ν cm^{-1}: 3392, 1718. ^1H and ^{13}C NMR data, see Tables 1 and 2.

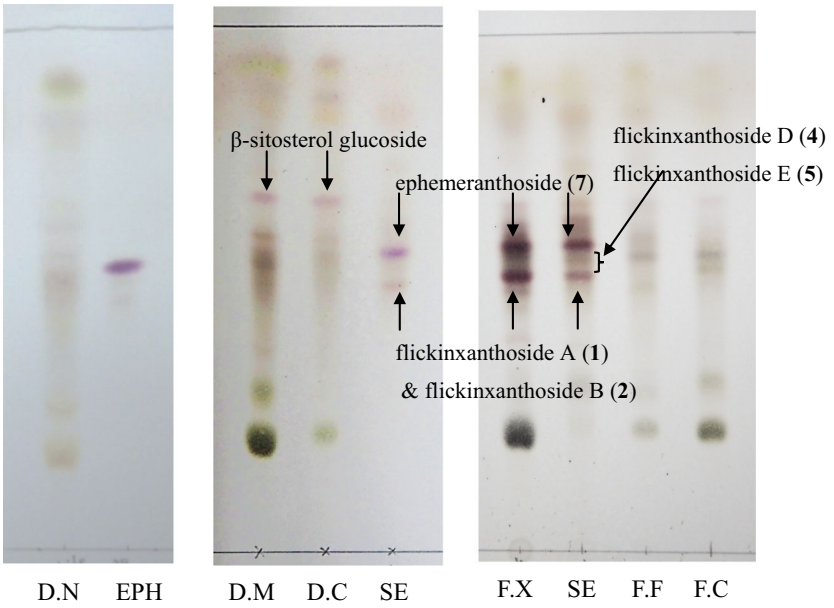

Fig. 7 Thin layer chromatography (TLC) of MeOH extracts of *Dendrobium* and *Flickingeria* plants

D.N: *D. nobile*
EPH: Ephemeranthoside (**7**)
D.M: *D. moniliforme*
D.C: *D. tosaense* Makino (=*D. catenatum* Lindl.)
SE: *Sekkoku,* current Japanese commercial crude drug
F.X: *F. xantholeuca*
F.F: *F. fimbriata*
F.C: *F. comata*

Flickinxthanthoside B (2)

A colorless powder, α_D^{25} −37.3 (c = 0.87, MeOH), HR-positive FAB-MS *m/z*: 515.2863, $[M+H]^+$, calcd. 515.2857 ($C_{26}H_{43}O_{10}$), IR ν cm^{-1}: 3400, 1714. ^1H and ^{13}C NMR data, see Tables 1 and 2.

Flickinxthanthoside C (3)

A colorless powder, α_D^{25} −34.6 (c = 0.46, MeOH), HR-positive FAB-MS *m/z*: 535.2527, $[M+Na]^+$, calcd. 535.2519 ($C_{26}H_{40}O_{10}Na$), IR ν cm^{-1}: 3399, 1715. ^1H and ^{13}C NMR data, see Tables 1 and 2.

Flickinxthanthoside D (4)

A colorless powder, α_D^{25} −66.3 (c = 0.28, MeOH), HR-positive FAB-MS *m/z*: 627.3386, $[M+H]^+$, calcd. 627.3381 ($C_{32}H_{51}O_{12}$), IR ν cm^{-1}: 3434, 1669. ^1H and ^{13}C NMR data, see Tables 1 and 2.

Flickinxthanthoside E (5)

A colorless powder, α_D^{25} −72.4 (c = 0.04, MeOH), HR-positive FAB-MS *m/z*: 651.3343, $[M+Na]^+$, calcd. 651.3356 ($C_{32}H_{52}O_{12}Na$), IR ν cm^{-1}: 3399, 1699. ^1H and ^{13}C NMR data, see Tables 1 and 2. The aglycone: 1,2-dehydroabbeokutone [10, 11], CD (EtOH) λmax nm (Δε) 238 (–5.82), 342 (+1.55).

Flickinxthanthol A (6)

A colorless powder, α_D^{25} −26.4 (c = 0.41, MeOH), HR-positive FAB-MS *m/z*: 337.2380, $[M+H]^+$, calcd. 337.2378 ($C_{20}H_{33}O_4$), IR ν cm^{-1}: 3411. ^1H and ^{13}C NMR data, see Tables 1 and 2.

Acid hydrolysis of 1–4

The glycoside (4 mg) was dissolved in 5 M HCl–CH_3OH (1:3) 5 ml, and the solution was refluxed in a water bath for 2 h. After cooling, the reaction mixture was passed through an Amberlite IRA-410 (OH^- form) column to neutralize it. The resulting solution was chromatographed on a Sep-Pak C-18 column, and elution with water afforded monosaccharides. Sugars were analyzed by TLC [*n*-propanol–H_2O (85:15)] and HPLC [column: Cosmosil Sugar-D (Nacalai Tesque, Kyoto, Japan), 4.6 mm i.d. × 250 mm; mobile phase, 75 % CH_3CN; flow rate, 0.8 mL/min; detection, RI; temperature, ambient]. D-glucose: α_D^{25} +57.8° (c = 0.04); t_R = 10.0 min (HPLC); Rf = 0.40 (TLC). L-Rhamnose: α_D^{25} +10.4° (c = 0.03); t_R = 6.8 min (HPLC); Rf = 0.63 (TLC).

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