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Isolation and characterization of related impurities in 24-epibrassinolide

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ABSTRACT

Two extra-hydroxylated analogs of 24-epibrassinolide (**2**), 17-hydroxy-24-epibrassinolide (**4**), 25-hydroxy-24-epibrassinolide (**5**), and two oxo-analogs of 24-epibrassinolide 22-oxo-23*S*-24-epibrassinolide (**6**), 22*R*-23-oxo-24-epibrassinolide (**7**) as impurities were isolated and identified from the initial material [purity: **2** and its 22*S*, 23*S*-isomer (**3**) >95%] by using various chromatographic methods and repeated crystallization. Among them, compound **4** and compound **6** were first reported. Their structures were established by spectrometric analysis and X-ray crystallography study. Formation of mixed crystals of **6** and **7** in the crystallization process was postulated and further confirmed.

1. Introduction

24-Epibrassinolide (**2**) is intensively investigated, because it is easier to synthesize[1–3] but has similar activity compared with brassinolide[4] (**1**) (Fig. 1), which is a powerful steroidal plant growth regulator.[5–8] On the other hand, extra-hydroxylated analogs as assay. Instead, the derivatives are important to the deactivation process of brassinasteroids.[14,15] In our study, two extra-hydroxylated analogs of 24-epibrassinolide, 17-hydroxy-24-epibrassinolide (**4**), 25-hydroxy-24-epibrassinolide (**5**), and two oxo-analogs of 24-epibrassinolide 22-oxo-23*S*-24-epibrassinolide (**6**), 22*R*-23-oxo-24-epibrassinolide (**7**) as impurities were isolated and identified

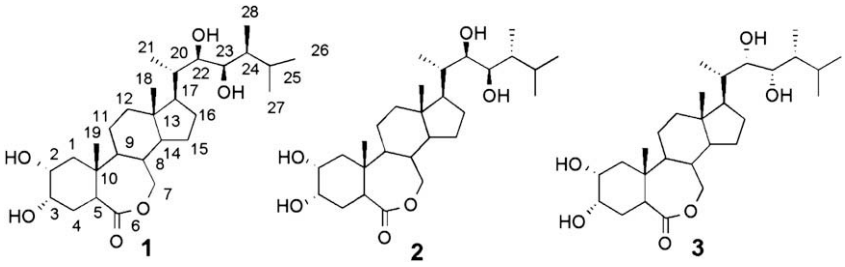

Figure 1. Brassinolide and its isomer.

metabolite in plant cell cultures[9–14] and oxo-analogs as endogenous materials[15,16] arouse particular interest to the researchers. Hydroxylation at C-25 and dehydrogenation at C-23 of brassinosteroids do not enhance activity in the rice leaf lamina inclination from the initial material [purity: **2** and its 22*S*, 23*S*-isomer (**3**) >95%] by using various chromatographic methods and repeated crystallization. Among them, compound **4** and compound **6** were first reported. Their structures were established by spectrometric analysis and X-ray crystallography study. Formation of mixed crystals of **6** and **7** in the crystallization process was postulated and further confirmed by X-ray crystallographic data.

HPLC analysis of brassinosteroids using a labeling technique with boronic acid has been intensively investigated since the

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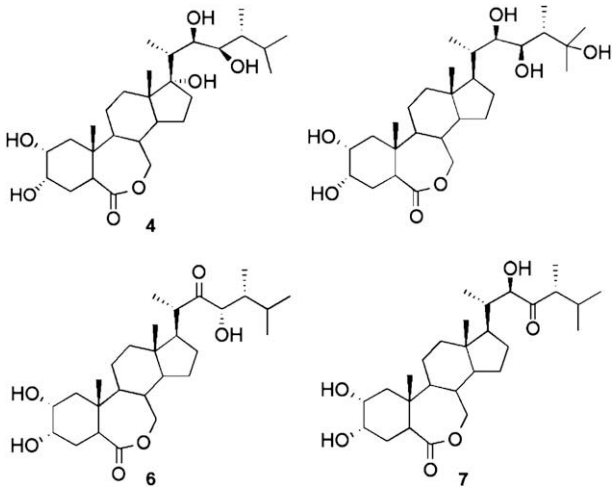


Figure 2. Structures of compounds **4**–**7**.

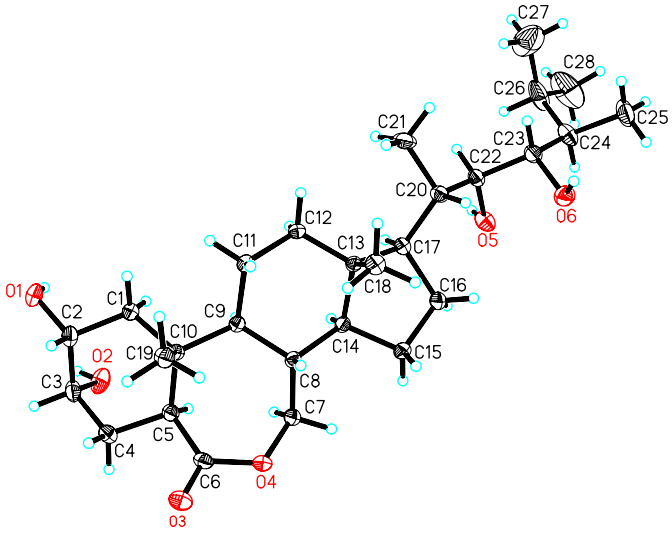


Figure 3. X-ray crystal structure of **3**.

brassinosteroids are lack of enough UV active chromophore[17] and the resulting boronate derivatizations can be detected with UV at 280 nm.[18–20] However, the methods are selective analytical methods of brassinosteroids and are not suitable for the purity analysis. Then, we have found HPLC analysis with ELSD detection is an effective method. And the ratios of all the compounds were determined by HPLC determination using the developing method.

The study may be helpful to conduct further research on biological activities and the structure–activity relationship of brassinosteroids and be useful in the quality control of the production of 24-epibrassinolide. Here we report the isolation, structure elucidation and spectral data of the compounds (Fig. 2).

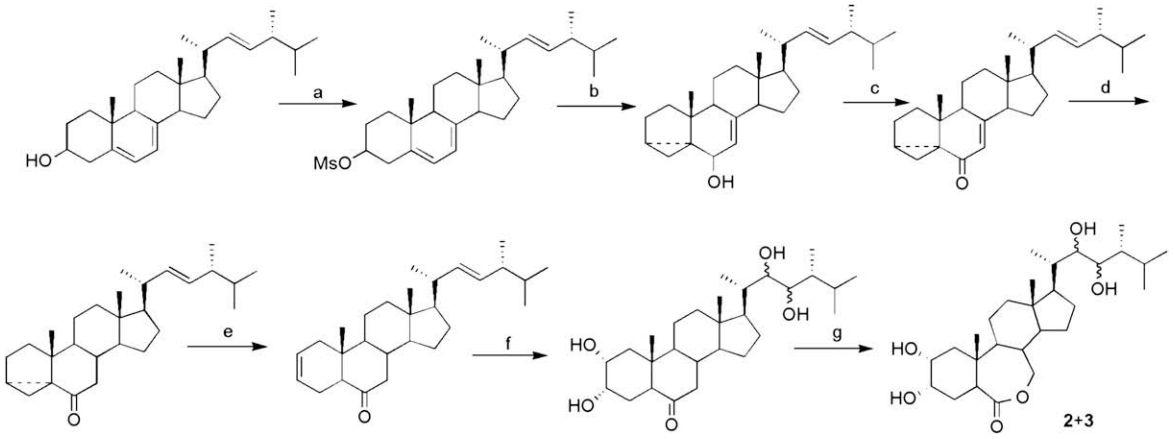
2. Results and discussion

The 24-epibrassinolide samples were purchased from Shanghai Vegcides Bio-Farm (Nanchang) Co., Ltd. China as powder and the method used for the synthesis of 24-epibrassinolide was shown in Scheme 1.

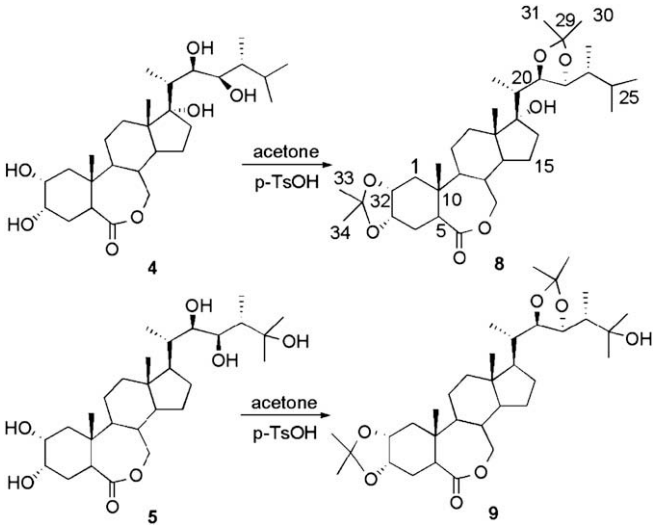
Fifty grams of the powder was subjected to column chromatography and repeated crystallization. Finally, compound **2** and its isomer **3** were obtained as colorless crystals from methanol and acetone, respectively. Their structures were confirmed by comparing their spectral data and melting points with the reported values.[21,22] In addition, we firstly gave the X-ray crystallographic data of **3** (Fig. 3).

The mixture of **4** and **5** was isolated as white powder. Then they were submitted to acetonides formation and isolated as **8** and **9** through chromatography (Scheme 2). Subsequently, compound **8** was obtained as colorless crystals from acetonitrile. Its molecular formula $C_{34}H_{56}O_7$ was supported by HRESIMS of the $[M+Na]^+$ and ion peak at m/z 577.4. In the ^{13}C NMR spectrum, 34 carbon signals were observed, constituted by 10 methyls, 7 methylenes, 11 methynes, 6 quaternary carbons (including 1 ester carbonyl). Analysis of ^1H NMR (Table 2) and ^{13}C NMR (Table 1) data of **8** and comparison with those of **2** showed that they possessed the same skeletons except for an additional hydroxy, which attached to a quaternary carbon. The position of the additional hydroxy was concluded on basis of the HMBC experiments: key correlations were observed between H-18, H-21, H-22, and C-17 (quaternary carbon, δ: 85.4) (Fig. 4). This was confirmed by HSQC and ^1H–^1H cosy experiments. The structure of **8** was completely established by X-ray analysis of a single crystal (Fig. 5).

Compound **9**, colorless powder, had a molecular formula $C_{34}H_{56}O_7$ determined by HRESIMS of the $[M+Na]^+$ and ion peak at m/z 577.4. The ^1H NMR showed that 26-H and 27-H were moved to low-field region (δ: 1.21, 1.26). Combining the ^{13}C NMR, the structure of **9** was concluded to be 2,3,22,23-tetra-*O*-methyl-25-



Scheme 1. Reagents and conditions: (a) CH_3SO_3Cl, Py; (b) $KHCO_3$, acetone, H_2O; (c) CrO_3, Py; (d) Li, NH_3, THF; (e) Py/HCl, LiBr, DMF; (f) OsO_4, NMO; (g) CF_3CO_3H, $CHCl_3$.



Scheme 2. The acetonides formation of **4** and **5**.

hydroxy-24-epibrassinolide. This assumption was subsequently confirmed by comparison of the spectra data with the reported values.[11]

The mixture of **6** and **7** was isolated as white powder. Although the mixture presented a single spot on TLC (silica gel) developed in several solvent systems, its ^{1}H NMR indicated this was a 3.6:1 mixture of two compounds. Then, we directed our attention to isolate and purify them by crystallization. Many solvent systems were attempted to crystallization and methanol and acetonitrile were considered to be suitable ones, finally. However, mixed crystals formed in the crystallization process either from methanol (the ratio of the two compounds was about 6:4) or from acetonitrile (the ratio of the two compounds was about 6:4). In such instances, we concluded that similarity between the two compounds led to the formation of mixed crystals.[23,24] The conclusion was confirmed since we obtained the structure of the two compounds by X-ray analysis (Fig. 6). Compound **7** could be incorporated into the crystal lattice of compound **6** and formed mixed crystals. Moreover, we got the pure compound **6** by repeated crystallization of the mother liquor. The molecular formula $C_{28}H_{46}O_6$ of **6** was supported by HRESIMS of the $[M+Na]^+$ and ion peak at m/z 479.3 $[M+H]^+$, 501.2 $[M+Na]^+$. The position of the carbonyl was justified by HMBC experiments: key correlation between the H-17, H-20, H-21, H-23, and the carbonyl (C-22) (Fig. 7). The ^{1}H (Table 2), ^{13}C NMR (Table 1), HSQC, and HMBC data led to assignment of all H- and C-atoms. Meanwhile, analysis of the spectra data (^{1}H, ^{13}C NMR, HSQC, and HMBC) of the mixture and comparison with the pure one (**6**), the structure of **7** could be fully exploited. The appearance of impurity **4**–**7** was likely to be attributed to overoxidation since CrO_3, OsO_4/NMO, and CF_3CO_3H were used in the synthesis of 24-epibrassinolide.

For analysis of the composition of all the compounds, the HPLC analysis was carried out on kromasil C18, 5 μm, 150×4.6 mm column using a mobile phase consisting of CH_3CN/H_2O (10:90 to 100:0) with ELSD detection at a flow rate of 1.0 ml/min at 40 °C. The result of the ratios of all the compounds was shown in Table 3.

Table 1
^{13}C NMR data of 24-epibrassinolide (**2**) and compounds **3**, **6**–**9**[a]

C	2[c]	3[c]	6	7	8	9
1	41.2	41.4	41.3	41.4	33.6	33.4
2	67.9	68.0	68.0	68.0	72.9	73.0
3	68.0	68.1	68.0	68.0	72.3	72.4
4	31.1	31.0	31.0	31.0	27.6	27.6
5	40.9	40.9	40.8	40.8	40.2	40.1
6	176.8	176.4	176.4	176.3	176.6	176.5
7	70.5	70.5	70.2	70.4	71.1	71.0
8	39.1	39.1	39.1	39.2	39.7	39.3
9	58.0	58.2	58.0	58.0	54.2	54.5
10	38.2	38.3	38.2	38.3	35.9	35.9
11	22.2	22.2	22.1	22.1	22.8	22.9
12	39.6	39.6	39.3	39.3	38.2	39.6
13	42.4	43.1	43.1	42.4	47.6	43.8
14	51.2	51.0	50.4	51.3	45.9	51.4
15	24.7	25.0	24.8	24.6	32.1	24.8
16	27.6	27.8	26.9	28.0	23.8	28.1
17	52.5	52.5	53.6	52.1	85.4	52.9
18	11.5	11.7	11.8	11.8	14.7	11.6
19	15.3	15.4	15.4	15.4	19.4	19.5
20	40.1	41.8	43.7	38.4	39.5	37.7
21	12.3	14.1	15.8	12.8	8.4	13.7
22	72.4	73.3	216.7	79.6	82.3	79.3
23	76.0	70.4	79.8	216.4	80.3	75.3
24	41.4	43.8	41.0	47.4	43.2	43.0
25	26.9	29.8	31.3	31.7	27.8	73.0
26[b]	22.1	21.5	20.9	20.9	20.9	28.8
27[b]	17.2	18.8	20.6	18.9	16.0	27.8
28	10.8	9.9	11.0	12.2	9.8	8.2
29					108.4	107.0
30[b]					27.3	27.3
31[b]					26.9	27.1
32					107.5	107.5
33[b]					26.6	26.6
34[b]					23.6	23.6

[a] Data were recorded at 75 MHz on a Bruker AM-300 spectrometer in $CDCl_3$ (δ in ppm).
[b] May be reversed.
[c] See Ref. 22.

3. Conclusion

Two extra-hydroxylated analogs of 24-epibrassinolide (**2**), 17-hydroxy-24-epibrassinolide (**4**), 25-hydroxy-24-epibrassinolide (**5**), and two oxo-analogs of 24-epibrassinolide 22-oxo-23*S*-24-epibrassinolide (**6**), 22*R*-23-oxo-24-epibrassinolide (**7**) as impurities were isolated and identified from the initial material [purity: **2** and its 22*S*, 23*S*-isomer (**3**) >95%] by using various chromatographic methods and repeated crystallization. Their structures were established by spectrometric analysis and X-ray crystallography study. Formation of mixed crystals of **6** and **7** in the crystallization process was postulated and further confirmed. The results of HPLC analysis showed the ratios of all the compounds. The study may be helpful to conduct further research on biological activities and the structure–activity relationship of the brassinosteroids and be useful in the quality control of the production of 24-epibrassinolide.

4. Experimental

4.1. General

1D and 2D NMR spectra were recorded on a Bruker AM-300 spectrometer in $CDCl_3$ or CD_3OD solution. MS were recorded on a Finnigan MAT-711 mass spectrometer. X-ray crystallographic data were obtained on a CAD-4 diffractometer. Column chromatography was performed on silicon gel (Qingdao Marine Chemical Co., Ltd., China; 300–400 mesh).

4.2. Isolation

Fifty grams of the powder was subjected to column chromatography (silica gel, 300–400 mesh, CH_2Cl_2/CH_3OH 30:1→4:1) to get four (1–4) fractions. Fraction 1 was subjected to repeated chromatography (CH_2Cl_2/CH_3OH 20:1→10:1), followed by

Table 2
^{1}H NMR data of 24-epibrassinolide (**2**) and compounds **3**, **6**–**9** in $CDCl_3$ (δ in ppm, J in Hz)[a]

H	2[c]	3[c]	6	7	8	9
1	1.86/1.55[d]	1.86/1.55[d]	1.84/1.54[d]	1.86/1.58[d]	2.32 (dd, 15.8, 3.0)/—[e]	2.28/1.09[d]
2	3.65 (br s)	3.70 (br s)	3.69 (br d, 10.8)	3.68 (br d, 8.5)	4.35 (br s)	4.35 (br s)
3	3.98 (br s)	4.01 (br s)	3.99 (br s)	3.99 (br s)	4.37 (br s)	4.35 (br s)
4	2.10/1.93[d]	2.13/1.93[d]	2.12/1.92[d]	2.14/1.95[d]	1.81/—[d,e]	1.78/—[d,e]
5	3.12 (dd, 11.8, 4.0)	3.11 (dd, 11.9, 4.3)	3.11 (dd, 11.9, 4.2)	3.11 (dd, 11.9, 4.2)	3.31 (t, 7.2)	3.28 (dd, 9.0, 5.4)
7	4.10 (br s)	4.08 (br d, 4.2)	4.05 (br m)	4.07 (br m)	4.11 (br m)	4.16 (br m)
8	1.72[d]	1.72[d]	1.69[d]	1.73[d]	1.82[d]	1.80[d]
9	1.30[d]	1.28[d]	1.27[d]	1.28[d]	1.79[d]	1.70[d]
11	1.80/1.40[d]	1.78/1.39[d]	1.78/1.39[d]	1.81/1.38[d]	1.88/1.34[d]	1.81/—[d,e]
12	1.99/1.22[d]	2.03/1.19[d]	1.95/1.30[d]	1.93/1.26[d]	1.93/1.85	2.00/1.22[d]
14	1.18[d]	1.12[d]	1.12[d]	1.23[d]	2.01[d]	—[e]
15	1.68/1.22[d]	1.68/1.25[d]	1.65/1.24[d]	1.74/1.24[d]	1.96/1.62[d]	1.62/—[d,e]
16	1.34/1.25[d]	1.77/1.42[d]	1.64/1.24[d]	2.08[d]	1.67/1.30[d]	1.26/—[d,e]
17	1.56[d]	1.23[d]	1.60[d]	1.64[d]		1.16[d]
18	0.71 (s)	0.72 (s)	0.72 (s)	0.75 (s)	0.75 (s)	0.71 (s)
19	0.92 (s)	0.91 (s)	0.89 (s)	0.90 (s)	0.88 (s)	0.88 (s)
20	1.46[d]	1.73[d]	2.66 (m)	1.90[d]	1.89[d]	1.92[d]
21	0.97 (d, 6.4)	1.01 (d, 6.6)	1.06 (d, 6.9)	0.70 (d, 6.7)	1.02 (d, 6.7)	0.99 (d, 6.6)
22	3.66 (br s)	3.56 (br s)		4.20 (br s)	4.14 (br m)	3.80 (dd, 8.9, 4.1)
23	3.37 (br d, 5.2)	3.70 (br s)	4.26 (br s)		3.62 (t, 8.2)	4.18 (br d, 8.8)
24	1.48[d]	1.30[d]	1.61[d]	2.51 (m)	1.58[d]	1.50[d]
25	1.90[d]	1.69[d]	1.72[d]	1.89[d]	2.08 (m)	
26[b]	0.92 (d, 6.7)	0.96 (d, 7.0)	1.04 (d, 6.3)	1.04 (d, 6.8)	0.93 (d, 6.7)	1.26 (d, 6.8)
27[b]	0.87 (d, 6.7)	0.87 (d, 7.0)	0.97 (d, 6.5)	0.86 (d, 6.8)	0.82 (d, 7.1)	1.21 (d, 6.6)
28	0.85 (d, 6.4)	0.89 (d, 6.3)	0.67 (d, 6.6)	1.01 (d, 6.8)	0.72 (d, 6.9)	0.99 (d, 6.6)
30[b]					1.35 (s)	1.35 (s)
31[b]					1.37 (s)	1.32 (s)
33[b]					1.52 (s)	1.51 (s)
34[b]					1.31 (s)	1.29 (s)

[a] Data were recorded at 300 MHz on a Bruker AM-300 spectrometer.
[b] May be reversed.
[c] See Ref. 22.
[d] The signals were overlapped.
[e] The signal of the HMQC spectrum was too low to assign the proton signals.

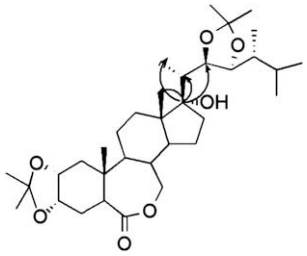


Figure 4. Key HMBC correlations of **8**.

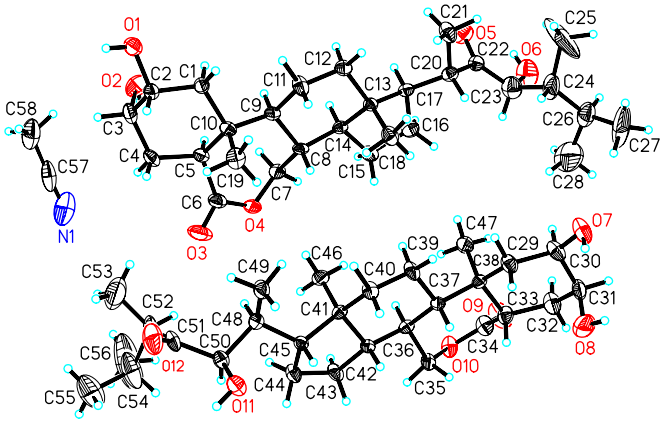


Figure 6. X-ray crystal structures of **6** and **7**.

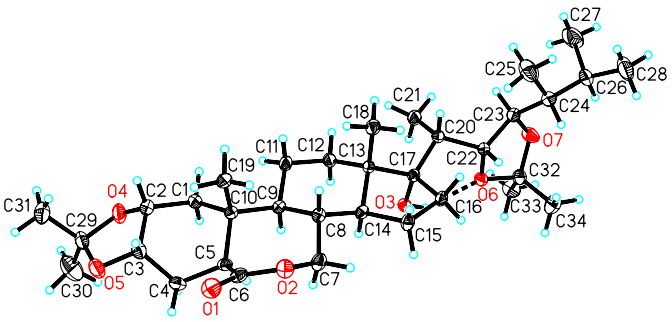


Figure 5. X-ray crystal structure of **8**.

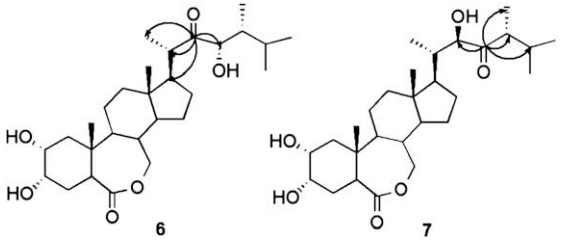


Figure 7. Key HMBC correlations of **6** and **7**.

Table 3
The ratios of all the compounds (%)

Compound	**2**	**3**	**4**	**5**	**6**	**7**
Ratio (%)	20.79	76.01	2.69	<0.1	0.39	0.11

repeated crystallization to yield compounds **3** (from acetone), **6** (from acetonitrile), and the mixture of compounds **6** and **7**. Fraction 2 was subjected to repeated chromatography (CH_2Cl_2/CH_3OH 10:1), followed by crystallization to yield compound **3** (from acetone) and compound **2** (from methanol). Fraction 3 was subjected to repeated chromatography (CH_2Cl_2/CH_3OH 8:1) to yield compound **2** and compound **4**.

Fraction 4 was dissolved in acetone, and treated with *p*-TsOH (0.1 equiv), at room temperature for 1 h. The mixture was poured into saturated $NaHCO_3$ solution and extracted with CH_2Cl_2. The organic phase was washed with water, brine and dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was subjected to column chromatography using hexane/AcOEt (4:1) as the eluent to afford compounds **8** and **9**.

4.3. Structure determination

The data of ^1H NMR, ^{13}C NMR of compounds **6**–**9** see Tables 1 and 2.

*4.3.1. 2,3,22,23-Tetra-O-methyl-17-hydroxy-24-epibrassinolide (**8**)*

ESIMS *m*/*z* 577.4 $[M+H]^+$; HRESIMS *m*/*z* 599.3918 $[M+Na]^+$, calcd for $C_{34}H_{56}O_7Na^{+1}$ *m*/*z* 599.3929.

*4.3.2. 22-Oxo-23S-24-epibrassinolide (**6**)*

ESIMS *m*/*z* 479.3 $[M+H]^+$, 501.2 $[M+Na]^+$; HRESIMS *m*/*z* 501.3187 $[M+H]^+$, calcd for $C_{28}H_{46}O_6Na^{+1}$ *m*/*z* 501.3179.

*4.3.3. X-ray structural analysis of **3** and **6**–**8***

Crystallographic data (excluding structural factors) for compounds **3**, **8** and the mixed crystals of **6** and **7** have been deposited with the Cambridge Crystallographic Data Centre (CCDC) as supplementary publication nos. CCDC 702652, CCDC 702653, and CCDC 702654. Copies of the data can be obtained free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, (fax: +44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

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