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Four new labdane-type diterpenoid glycosides from *Diplopterygium laevissimum*

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Abstract: Four new labdane-type diterpenoid glycosides, laevissiosides A–D (**1**–**4**) were isolated from the 95% ethanol extract of *Diplopterygium laevissimum* (Christ) Nakai, along with two known analogues, 18-*β*-D-glucopyranosyl ester-sclareol (**5**) and 18-hydroxy-sclareol (**6**). The structures of compounds **1**–**4** were elucidated by extensive 1D and 2D NMR spectroscopy as well as high-resolution MS analyses. All isolated compounds were evaluated for their cytotoxic effects.

Keywords: Diplopterygium laevissimum, labdane-type diterpenoid glycosides, laevissiosides

Introduction

Diplopterygium laevissimum (Christ) Nakai, belonging to the Gleicheniaceae family, is widely distributed in south China. Its rhizome has been used for treating hemostasis, stomach, and epistaxis as Chinese herbal medicine.[1] Many clerodane- and labdane-type diterpenoid glycosides, which are commonly glycosidated at C-13 in ferns, have been isolated from this family.[2–8] Previous research showed that some clerodane-type diterpenoid glycosides isolated from *Dicranopteris* species could accelerate the growth of the stems of lettuce and inhibit the root growth.[5]

Our previous chemical studies have led to the isolation of two highly oxygenated phenolic derivatives and some clerodane-type diterpenoid glycosides from *Dicranopteris* and an *ent*-kaurene diterpenoid glycoside from *Hicriopteris*.[6,9–11] As a systematic research work on the bioactive constituents from the ferns, the whole plant of *D. laevissimum* had been studied, which led to the isolation of four new labdane-type diterpenoid glycosides (**1**–**4**), along with two known analogues (**5** and **6**). All of these showed no *in vitro* cytotoxicity against five human cancer cell lines (HL-60, SMMC-7712, A-549, SK-BR-3 and PANC-1). Herein, the isolation and structure elucidation of compounds **1**–**4** were described.

Results and Discussion

Compound **1** was obtained as a white amorphous powder. The molecular formula $C_{35}H_{52}O_{10}$ was established by the HRESIMS (631.3471 $[M - H]^-$; calcd. 631.3482), corresponding to ten degrees of unsaturation. The IR spectrum showed the presence of hydroxyl (3428 cm^{-1}) and carbonyl (1704 cm^{-1}) groups. The ^1H and ^{13}C NMR (Tables 1 and 2) indicated the existence of a set of signals for a hexose [anomeric signals at δ_H 4.30 (d, J = 8.0 Hz); δ_C 104.6] and other 20 carbon resonances, including two olefinic carbons (δ_C 147.5 and 110.8) and three oxygen-bearing carbons (δ_C 73.4, 73.9 and 79.2). These data were very similar to those of 18-*β*-D-glucopyranosyl ester-sclareol (**5**), a known compound also isolated from this plant. However, detailed comparison the MS and NMR data of **1** with those of **5** revealed that **1** had one more *p*-coumaroyl group, which was attached to C-4′ of the sugar moiety as concluded from the HMBC (Figure 1) correlations of H-4′ (δ_H 4.84) with C-1″ (δ_C 167.4). The double bond of the *p*-coumaroyl group was suggested as *trans*- due to the coupling constant (J = 15.6 Hz). Acidic hydrolysis of **1** gave D-glucose as sugar residue. The coupling constants of the anomeric proton (J = 8.0 Hz) indicated the *β* configuration of glucosyl moiety. Assignment of glycosidic protons system was achieved by analysis of ^1H-^1H COSY and HSQC. The location

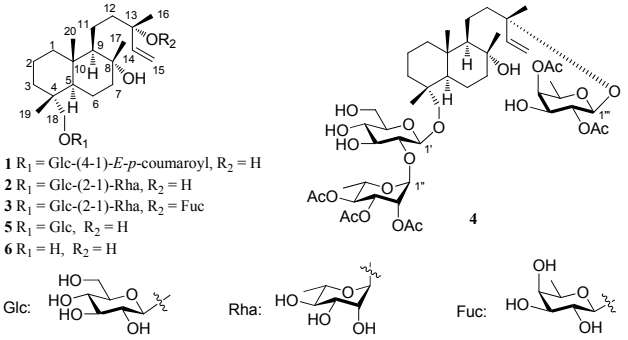

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Table 1. ^{1}**H NMR data of compounds 1–4 (*δ* in ppm, *J* in Hz)**

no.	**1**[a]	no.	**2**[a]	**3**[b]	**4**[a,c]
1	1.66 (m); 0.95 (m)	1	1.59 (m); 1.09 (m)	1.59 (m); 1.04 (m)	1.79 (m); 1.13 (m)
2	1.63 (m); 1.42 (m)	2	1.59 (m); 1.41 (m)	1.60 (m); 1.45 (m)	1.69 (m); 1.51 (m)
3	1.56 (m); 1.27 (m)	3	1.59 (m); 1.18 (m)	1.50 (m); 1.30 (m)	1.48 (m); 1.40 (m)
5	1.48 (m)	5	1.36 (m)	1.38 (m)	1.32 (m)
6	1.55 (m); 1.25 (m)	6	1.52 (m); 1.27 (m)	1.55 (m); 1.36 (m)	1.62 (m); 1.32 (m)
7	1.70 (m); 1.26 (m)	7	1.74 (m); 1.52 (m)	1.75 (m); 1.47 (m)	1.80 (m); 1.50 (m)
9	1.16 (m)	9	1.35 (m)	1.24 (m)	1.00 (m)
11	1.33 (m)	11	1.47 (m); 1.29 (m)	1.50 (m); 1.36 (m)	1.43 (m)
12	1.73 (m); 1.55 (m)	12	1.78 (m); 1.56 (m)	1.62 (m); 1.51 (m)	1.76 (m); 1.54 (m)
14	5.91 (dd, 10.8, 17.6)	14	5.89 (dd,10.5, 17.0)	5.91 (dd, 10.8, 17.6)	5.88 (dd, 10.8, 18.0)
15	4.92 (d, 10.8); 5.19 (d, 17.6)	15	5.15 (d, 17.0); 4.91(d, 10.5)	5.17 (d, 17.6); 5.15 (d, 10.8)	5.18 (m); 5.16 (m)
16	1.20 (s)	16	1.05 (s)	1.09 (s)	1.09 (s)
17	1.10 (s)	17	1.19 (s)	1.32 (s)	1.32 (s)
18	3.45 (m); 3.25 (m)	18	3.38 (m); 3.20 (m)	3.48 (m); 3.21 (m)	3.44 (m); 3.35 (m)
19	0.75 (s)	19	0.70 (s)	0.76 (s)	0.81 (s)
20	0.82 (s)	20	0.79 (s)	0.82 (s)	0.85 (s)
1′	4.30 (d, 8.0)	1′	4.27 (d, 7.5)	4.25 (d, 7.2)	4.42 (d, 7.2)
2′	3.33 (m)	2′	3.24 (m)	3.45 (m)	3.53 (m)
3′	3.68 (m)	3′	3.52 (m)	3.46 (m)	3.68 (m)
4′	4.84 (t, 9.6)	4′	3.26 (m)	3.24 (m)	3.36 (m)
5′	3.48 (m)	5′	3.48 (m)	3.19 (m)	3.35 (m)
6′	3.54 (m); 3.58 (m)	6′	3.79 (m); 3.62 (m)	3.64 (m); 3.83 (m)	3.68 (m); 3.84 (m)
2″	6.36 (d, 15.6)	1″	5.51 (d, 1.5)	5.42 (d, 1.5)	5.62 (d, 0.8)
3″	7.64 (d, 15.6)	2″	3.87 (m)	3.91 (m)	5.32 (m)
5″/9″	7.55 (d, 8.8)	3″	3.77 (m)	3.74 (m)	5.15 (m)
6″/8″	6.90 (d, 8.8)	4″	3.43 (m)	3.40 (m)	5.12 (m)
		5″	4.08 (m)	4.06 (m)	4.46 (m)
		6″	1.25 (d, 6.5)	1.27 (d, 6.0)	1.18 (d, 6.4)
		1‴		4.28 (d, 8.0)	4.52 (d, 8.0)
		2‴		3.41 (m)	5.37 (m)
		3‴		3.40 (m)	3.87 (m)
		4‴		3.55 (m)	4.92 (m)
		5‴		3.52 (m)	3.82 (m)
		6‴		1.20 (d, 6.4)	1.08 (d, 6.4)

[a]Measured in acetone-d_6. [b]Determined in CD_3OD. [c]Acetyl groups δ_H: (1.98, 2.06, 2.13, 2.13, 2.16).

of the sugar unit was established by the HMBC correlations of H-1′ (δ_H 4.30) with C-18 (δ_C 79.2).

The relative configuration of the aglycone was established on a ROESY experiment. The ROESY correlations (Figure 2) between H-5 and H-9 confirmed that these hydrogen atoms were *α*-oriented, while correlations of H-11/Me-17, H-11/Me-20, Me-17/Me-19, Me-17/Me-20, and Me-19/Me-20 indicated they were *β*-orientation. The absolute configuration of C-13 was inferred as *S* according to the chmical shift of C-13 (δ_C 73.4).[12–14] Therefore, the structure of **1** was determined as shown, named laevissioside A.

Compound **2**, a white amorphous powder, and its molecular formula, $C_{32}H_{56}O_{12}$, was determined on the basis of the HRESIMS (667.3454 $[M + Cl]^-$; calcd. 667.3460). The ^{1}H and ^{13}C NMR spectroscopic data of **2** were very similar to those of **5**, except for one more sugar moiety signals (δ_C 100.8, 72.0, 71.7, 73.7, 69.2, 18.3) presented in **2** which was further confirmed by mass spectra. Acidic hydrolysis of **2** gave D-glucose and L-rhamnose as sugar residues. The coupling

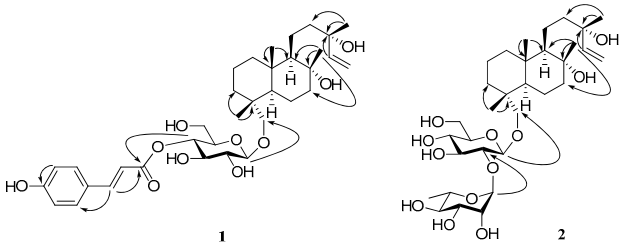

Figure 1. Selected HMBC correlations of compound **1** and **2**

constants (δ_H 4.27, J = 7.5 Hz and δ_H 5.51, J = 1.5 Hz) of anomeric protons of the two sugar moieties indicated the *β* configuration glucose and *α* configuration of rhamnose. The HMBC correlations between H-1″ (δ_H 5.51) and C-2′ (δ_C 77.5) identified a rhamnosyl (1→2) glucopyranosyl linkage. Furthermore, the sugar chain was linked to C-18 of the aglycone as inferred from the HMBC (Figure 1) correlation of

Table 2. ^{13}C NMR data of compounds 1-4 (δ in ppm)

no.	1[a]	no.	2[a]	3[b]	4[a,c]
1	39.8 CH_2	1	39.7 CH_2	40.4 CH_2	39.5 CH_2
2	18.5 CH_2	2	18.4 CH_2	18.8 CH_2	18.4 CH_2
3	36.6 CH_2	3	36.5 CH_2	37.0 CH_2	36.4 CH_2
4	37.9 C	4	37.8 C	38.3 C	38.2 C
5	49.6 CH	5	48.9 CH	50.7 CH	51.2 CH
6	21.0 CH_2	6	20.9 CH_2	21.4 CH_2	21.5 CH_2
7	44.6 CH_2	7	44.7 CH_2	44.8 CH_2	45.4 CH_2
8	73.9 C	8	74.6 C	75.4 C	73.8 C
9	62.1 CH	9	60.9 CH	62.0 CH	62.9 CH
10	40.0 C	10	40.0 C	40.4 C	40.0 C
11	20.1 CH_2	11	20.4 CH_2	20.5 CH_2	19.9 CH_2
12	46.5 CH_2	12	45.7 CH_2	44.8 CH_2	46.7 CH_2
13	73.4 C	13	73.7 C	81.8 C	80.7 C
14	147.5 CH	14	147.0 CH	145.0 CH	143.5 CH
15	110.8 CH_2	15	111.1 CH_2	115.2 CH_2	115.5 CH_2
16	28.1 CH_3	16	23.6 CH_3	23.0 CH_3	23.8 CH_3
17	24.6 CH_3	17	27.3 CH_3	23.7 CH_3	24.6 CH_3
18	79.2 CH_2	18	78.2 CH_2	79.4 CH_2	79.0 CH_2
19	17.9 CH_3	19	18.2 CH_3	18.2 CH_3	17.9 CH_3
20	16.2 CH_3	20	16.2 CH_3	16.5 CH_3	16.3 CH_3
1′	104.6 CH	1′	102.9 CH	103.5 CH	102.5 CH
2′	75.2 CH	2′	77.5 CH	78.0 CH	75.9 CH
3′	75.5 CH	3′	79.6 CH	79.8 CH	79.3 CH
4′	72.3 CH	4′	71.9 CH	72.0 CH	72.1 CH
5′	75.8 CH	5′	76.8 CH	77.9 CH	77.5 CH
6′	62.4 CH_2	6′	62.6 CH_2	62.7 CH_2	62.5 CH_2
1″	167.4 C	1″	100.8 CH	101.5 CH	97.8 CH
2″	115.2 CH	2″	72.0 CH	72.3 CH	70.5 CH
3″	146.1 CH	3″	71.7 CH	72.1 CH	71.0 CH
4″	121.8 C	4″	73.7 CH	74.0 CH	74.0 CH
5″/9″	116.7 CH	5″	69.2 CH	70.0 CH	67.1 CH
6″/8″	131.0 CH	6″	18.3 CH_3	18.8 CH_3	17.6 CH_3
7″	160.8 C	1‴		99.6 CH	97.1 CH
		2‴		72.4 CH	70.1 CH
		3‴		75.2 CH	71.1 CH
		4‴		73.0 CH	73.4 CH
		5‴		71.5 CH	69.5 CH
		6‴		17.0 CH_3	16.9 CH_3

[a]Measured in acetone-d_6. [b]Determined in CD_3OD. [c]Acetyl groups (δ_C: C=O 170.0, 170.4, 170.6, 171.0, 171.5; Me: 20.7, 20.8, 21.2, 21.2, 21.2).

H-1′ (δ_H 4.27) with C-18 (δ_C 78.2). Thus, the structure of **2** was elucidated and named as laevissioside B.

Compound **3**, obtained as a white amorphous powder, had the molecular formula $C_{38}H_{66}O_{16}$ as determined by the analysis of ^{1}H, ^{13}C, and DEPT NMR data and verified by the HRESIMS (813.4042 $[M + Cl]^-$; calcd. 813.4039). Compared with **2**, compound **3** had one more sugar moiety (δ_C 99.6, 72.4, 75.2, 73.0, 71.5, and 17.0) which was attached to C-13 as inferred from the HMBC correlations from H-1‴ to C-13. Acidic hydrolysis of **3** gave D-glucose, L-rhamnose, and D-fucose as sugar residues. They were in β, α and β configurations, respectively, by the coupling constants (δ_H 4.25, J = 7.2 Hz, δ_H 5.42, J = 1.5 Hz, and δ_H 4.28, J = 8.0 Hz) of their anomeric protons. The sugar moiety linked to C-13 was supposed to be fucose by the HMBC correlations of the anomeric proton H-1‴ (δ_H 4.28) with C-13 (δ_C 81.8). The linkage and location of the other two sugar moieties were suggested the same as compound **2** deduced from the HMBC correlations. Accordingly, the structure of **3** was established as shown, named laevissioside C.

The molecular formula of compound **4** was deduced as $C_{48}H_{76}O_{21}$ by the HRESIMS (1023.4557 $[M + Cl]^-$; calcd 1023.4567). The ^{1}H and ^{13}C NMR features of **4** were closely related to those of **3**. The only difference was that there were five more acetyl groups in **4**. The locations of acetyl groups were confirmed by the HMBC experiments. The correlations of H-2‴ (δ_H 5.37) with δ_C 170.4, H-4‴ (δ_H 4.92) with δ_C 170.0, H-2″ (δ_H 5.32) with δ_C 171.5, H-3″ (δ_H 5.15) with δ_C171.0, and H-4″ (δ_H 5.12) with δ_C 170.6 indicated the five acetyl groups attached to C-2‴, C-4‴, C-2″, C-3″, and C-4″, respectively. Therefore, the structure of **4** was identified and named as laevissioside D.

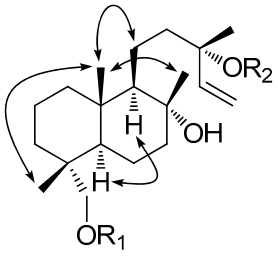

Figure 2. Significant ROESY correlations of compounds **1**–**4**

Since the known compounds 18-β-D-glucopyranosyl ester-sclareol (**5**, $[\alpha]_D^{24.3}$ – 27.7) and 18-hydroxy-sclareol (**6**, $[\alpha]_D^{24.3}$ – 4.7), whose physical properites were quite difference with that reported,[15,16] were also isolated from this plant, compounds **1**–**4** should be labdane-type diterpenoid glycosides from the biogenic view.

All compounds isolated were evaluated for their cytotoxic activity against five human cancer cell lines, HL-60 myeloid leukemia, SMMC-7721 hepatocellular carcinoma, A-549 lung cancer, SK-BR-3 breast cancer, PANC-1 pancreatic cancer, applying the MTT method. However, all of the compounds were inactive, and they showed IC_{50} values > 40 μM.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Horiba SEPA-300 polarimeter. IR spectra were obtained by Tensor 27 FT-IR spectrometer with KBr pellets. The ^{1}H and ^{13}C NMR spectra were recorded on Bruker AV-400 spectrometers in acetone-d_6 at room temperature (δ in ppm, J in Hz). FABMS was carried out on a VG Autospec-3000 spectrometer. HRESIMS was recorded with an API QSTAR Pulsar i spectrometer. Silica gel (200–300 mesh), Silica gel H (Qingdao Marine Chemical Ltd., China), and LiChroprep RP-18 silica gel (40–63 μm, Merck, Dramstadt, Germany) were used for column chromatography. Fractions were monitored by TLC and spots visualized by heating silica gel plates immersed with 15% H_2SO_4 in ethanol. Solvents were distilled prior to use. Preparative HPLC was performed on a Shimadzu

LC-8A preparative liquid chromatograph with Shimadzu PRC-ODS (K) column. Sephadex LH-20 (Amersham Pharmacia biotech, Sweden).

Plant Material. The aerial parts of *D. laevissimum* were collected from Pingbian, Yunnan Province, China in July 2007 and identified by Professor Xiao Cheng at Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No. 200707A03) has been deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. The dried and powdered plant materials (2.6 kg) were extracted with 95% ethanol (15.0 L, each 2 d) for three times. After evaporation of the solvent in vacuo, the concentrate was suspended into H_2O and partitioned successively with ethyl acetate. The ethyl acetate extract (120 g) was chromatographed on a silica gel column eluted with $CHCl_3$-MeOH (1:0 to 5:5) to give five fractions 1–5. Fraction 2 was subjected to column chromatograph (CC) over silica gel (petroleum ether-acetone 8.5:1.5) and further purified by recrystallization to obtain **6** (20 mg). Fraction 3 was eluted with $CHCl_3$-MeOH (9:1) over silica gel CC then further purified by RP-18 and Sephadex LH-20 to yield **1** (3 g), **4** (500 mg), and **5** (2 g). Fraction 4 was subjected to ($CHCl_3$:MeOH = 8.5:1.5) and further purified by RP-18 and Sephadex LH-20 to afford **2** (8 mg) and **3** (20 mg).

Laevissioside A: amorphous powder; $[\alpha]_D^{19.2}$ – 50.4 (*c* = 0.14, MeOH). UV (MeOH) λ_{max} (log ε): 314 (4.18), 227 (3.94), 211 (3.95), 200 (3.99), 192 (3.91) nm. IR (KBr): 3428, 2931, 1704, 1630, 1604, 1515, 1450, 1387, 1162, 1066, 1031 cm^{-1}. ^1H and ^{13}C NMR: see Table 1. FABMS (neg.) *m/z*: 631 [M – H]$^-$. HRESIMS (neg.) *m/z*: 631.3471 ($C_{35}H_{51}O_{10}$; calcd. 631.3482).

Laevissioside B: amorphous powder; $[\alpha]_D^{26.4}$ – 62.2 (*c* = 0.18, MeOH). UV (MeOH) λ_{max} (log ε): 203 (3.38), 194 (3.12) nm. IR (KBr): 3423, 2927, 1069, 1052 cm^{-1}. ^1H and ^{13}C NMR: see Table 2. FABMS (neg.) *m/z*: 631 [M – H]$^-$. HRESIMS (neg.) *m/z*: 667.3454 ($C_{32}H_{56}O_{12}Cl$; calcd. 667.3460).

Laevissioside C: amorphous powder; $[\alpha]_D^{18.9}$ – 11.2 (*c* = 0.10, MeOH). UV (MeOH) λ_{max} (log ε): 201 (3.31), 191 (3.10) nm. IR (KBr): 3431, 2926, 1704, 1638, 1384, 1169, 1128, 1054 cm^{-1}. ^1H and ^{13}C NMR: see Table 2. FABMS (neg.) *m/z*: 777 [M – H]$^-$, 631 [M – 146 – H]$^-$. HRESIMS (neg.) *m/z*: 813.4042 ($C_{38}H_{66}O_{16}Cl$; calcd. 813.4039).

Laevissioside D: amorphous powder; $[\alpha]_D^{26.6}$ – 58.3 (*c* = 0.25, MeOH). UV (MeOH) λ_{max} (log ε): 199 (3.07), 192 (3.12) nm. IR (KBr): 3442, 2935, 1749, 1373, 1228, 1062, 1062 cm^{-1}. ^1H and ^{13}C NMR: see Table 2. Acetyl groups (δ_C: C=O 170.0, 170.4, 170.6, 171.0, 171.5; Me: 20.7, 20.8, 21.2, 21.2, 21.2) δ_H: (1.98, 2.06, 2.13, 2.13, 2.16). FABMS (neg.) *m/z*: 987 [M – H]$^-$. HRESIMS (neg.) *m/z*: 1023.4557 ($C_{48}H_{76}O_{21}Cl$; calcd. 1023.4567).

Acidic Hydrolysis of Compounds 1–3. Compounds **1**–**3** (6–8 mg) were hydrolyzed with 2 M HCl-dioxane (1:1, 4 mL) under reflux for 6 h. The reaction mixture was extracted with $CHCl_3$ five times (4 mL × 5). The aqueous layer was neutralized with 2 M $NaHCO_3$, and was evaporated to dryness. The dry powders were dissolved in pyridine (2 mL). Then L-cysteine methyl ester hydrochloride (about 1.5 mg) was added and kept at 60 °C for 1 h. Next, trimethylsilylimidazole (about 1.5 mL) was added to the reaction mixture in ice water and kept at 60 °C for 30 min. The mixture was subjected to GC analysis, run on a Shimadzu GC-14C gas chromatograph equipped with a 30 m × 0.32 mm i.d. 30QC2/AC-5 quartz capillary column and an H_2 flame ionization detector with the following conditions: column temperature, 180–280 °C; programmed increase, 3 °C/min; carrier gas, N_2 (1 mL/min); injector and detector temperature, 250 °C; injection volume, 4 μL; and split ratio, 1/50. The configuration of D-glucose, L-rhamnose, and D-fucose were determined by comparison of the retention time of the corresponding derivatives with those of standard D-glucose, L-rhamnose, and D-fucose, giving a peak at 18.576, 16.173, and 14.865 min, respectively.

Cytotoxic Bioassay. The cytotoxicity assay was performed according to the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method,[17] by use of the following five human cancer cell lines: Human myeloid leukemia HL-60, hepatocellular carcinoma SMMC-7721, lung cancer A-549, breast cancer SK-BR-3, and pancreatic cancer PANC-1. The IC_{50} values were calculated by the Reed and Muench method.[18]

Acknowledgments

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