# Oleanane-Type Triterpene Saponins and Cassaine-Type Diterpenoids from *Erythrophleum fordii*

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**Key words**
- *Erythrophleum fordii* Oliver
- Leguminosae
- oleanane-type triterpene saponin
- cassaine-type diterpenoid
- cytotoxic activity

## Abstract

Phytochemical investigation of the EtOH extract of the leaves of *Erythrophleum fordii* led to the isolation of two oleanane-type triterpene saponins (**1**–**2**) and five cassaine-type diterpenoids (**4**–**8**) along with one known methyl 3*β*-hydroxy-erythrosuamate (**3**). Their structures were established by extensive NMR, as well as ESI-MS analyses and acid hydrolysis. Biological evaluation of compounds **3**–**8** against five human cancer cell lines revealed that compounds **5**–**7** exhibited potent cytotoxic activity with $IC_{50}$ values ranging from 1.51 to 8.68 µM.

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## Introduction

*Erythrophleum fordii* Oliver (Leguminosae) is a toxic plant which grows in South China, Taiwan, and Vietnam [1, 2]. Its bark has traditionally been used by the native Chinese for invigoration and promotion of blood circulation [3]. In previous phytochemical studies on the genus *Erythrophleum,* alkaloids (cassaine-type diterpenoid amines and amides containing a perhydrophenanthrene skeleton), triterpenoids, diterpenoids, and diterpenoid dimers have been isolated and identified [4–11]. The present investigation of the leaves of *E. fordii,* with particular attention paid to the terpenoids and their glycosides, resulted in the isolation of compounds **1**–**8**, including two new triterpene glycosides (**1**–**2**), five new diterpenoids (**4**–**8**), and one previously reported methyl 3*β*-hydroxy-erythrosuamate (**3**). To our knowledge, it is the first report of triterpene saponins from the *Erythrophleum* genus. The following describes the structural characterization of compounds **1**–**8** and the cytotoxic activities of compounds **5**–**7**.

## Materials and Methods

### General procedures

Melting points were determined on a XT-5B micromelting point apparatus and uncorrected. Optical rotations were measured with a P2000 polarimeter. IR spectra were recorded on a Nicolet 5700 FT-IR spectrometer by a microscope transmission method. UV spectra were obtained on a V650 spectrometer. 1D and 2D NMR experiments were performed on an Inova 500 FT-NMR spectrometer. ESIMS and HR-ESIMS were measured on an Agilent 1100 Series LC/MSD Trap mass spectrometer. Preparative HPLC was performed on a Shimadazu LC-6AD instrument with a SPD-10A detector, using an YMC-Pack ODS-A column (250 × 20 mm, 5 µm). Analytical HPLC was performed on an Agilent 1100 Series instrument with a DAD detector, using an YMC-Pack ODS (100 × 4.6 mm, 5 µm). GC analyses were obtained using an Agilent N6890 instrument. Macroporous resin HP 20 (Mitsubishi Chemical Corporation), polyamide (30–60 mesh; Jiangsu Linjiang Chemical Reagents Factory), Sephadex LH-20 (Amersham Pharmacia Biotech AB), and ODS (50 µm, Merck) were used for column chromatography. Silica gel GF-254 (Qingdao Marine Chemical Factory) was used for TLC. Solvents (petroleum, $CHCl_3$, MeOH, and EtOH) were analytical grade and purchased from Beijing Chemical Company. The authentic sugars, L-cysteine methyl ester hydrochrochloride, and *N*-trimethylsilylimidazole, were bought from Fluka.

## Plant material

The leaves of *Erythrophleum* fordii Oliver (Leguminosae) were collected from Guangxi Province, China, and identified by Prof. Songji Wei (Guangxi College of Chinese Traditional Medicine) in August 2007. A voucher specimen (NO. 07089) was deposited in the Herbarium of the Department of Medicinal Plants, Institute of Materia Medica, Chinese Academy of Medical Sciences.

## Extraction and isolation

The air-dried and powdered leaves (5.2 kg) were refluxed three times with 95% EtOH (3 h for each time). The combined EtOH extract was evaporated under reduced pressure to yield a dark brown residue (806 g, 15.5% yielded from the dried leaves), which was dissolved in MeOH and then applied to the top of the diatomite column (15 × 120 cm, 2000 g) and eluted successively with petroleum, EtOAc, and MeOH to yield three fractions (A–C, 90 g, 45 g, and 532 g, respectively) after removal of the solvents. Chemical screening including HPLC/UV/ESI$^n$ analysis of these three extracts indicated the presence of triterpene saponins and diterpenoids in fractions A and C. Fraction A (90 g) was directly chromatographed over a polyamide column (30–60 mesh, 10 × 120 cm, 1200 g) eluted with 30% EtOH (10 L) and 60% EtOH (10 L) to yield two corresponding fractions $A_1$ (11.5 g) and $A_2$ (18.3 g) after removing solvents. The fraction $A_1$ (11.5 g) eluted by 30% EtOH was subjected to Sephadex LH-20 (4 × 85 cm, 300 g) eluting with $CH_2Cl_2$: MeOH (2 L) to give three fractions ($A_{1-1}$ – $A_{1-3}$). Fraction $A_{1-2}$ (8.7 g) was submitted to ODS column (50 µm, 4 × 50 cm, 300 g) using gradient mixtures of MeOH – $H_2O$ (10 : 90, 1.5 L), (20 : 80, 1.5 L), (30 : 70, 2 L), (40 : 60, 2 L), (50 : 50, 2 L), (60 : 40, 2 L), (70 : 30, 2 L), (80 : 20, 1.5 L), and (90 : 10, 1.5 L) as eluants, to give ten subfractions ($A_{1-2-1}$ – $A_{1-2-10}$). Subfraction $A_{1-2-7}$ (360 mg) was purified by preparative HPLC using MeCN–$H_2O$ (35 : 65) to yield compound **5** (63 mg; flow rate: 5 mL/min; $t_R$ = 72.0 min). Subfraction $A_{1-2-9}$ (570 mg) was separated by preparative HPLC using MeCN–$H_2O$ (50 : 50) to yield compounds **6** (31 mg; flow rate: 6 mL/min; $t_R$ = 89.7 min) and **7** (15 mg; flow rate: 6 mL/min; $t_R$ = 62.0 min). Fraction C (532 g) was submitted to a polyamide column (30–60 mesh, 10 × 150 cm, 2500 g) using 40% EtOH (40 L) and 85% EtOH (20 L) as eluents; after evaporation of the solvent, fractions $C_1$ (360 g) and $C_2$ (15 g) were obtained. Fraction $C_1$ (360 g) was fractionated over macroporous resin (10 × 150 cm, 1800 g) with $H_2O$ (20 L), 35% EtOH (20 L) and 80% EtOH (20 L) to furnish three fractions $C_{1-1}$ (250 g), $C_{1-2}$ (170 g), and $C_{1-3}$ (20 g), respectively. Fraction $C_{1-3}$ (20 g) was chromatographed over Sephadex LH-20 (4 × 100 cm, 400 g) eluting with MeOH (2 L) to give three fractions ($C_{1-3-1}$ – $C_{1-3-3}$). Fraction $C_{1-3-1}$ (15.7 g) was submitted to ODS column (50 µm, 4 × 50 cm, 300 g) eluted with a system of MeOH–$H_2O$ (10 : 90, 20 : 80, 30 : 70, 40 : 60, 50 : 50, 60 : 40, 70 : 30, 80 : 20 and 90 : 10, each 1.5 L) to afford eleven subfractions ($C_{1-3-1-1}$ – $C_{1-3-1-11}$). Compound **3** (33 mg; flow rate: 6 mL/min; $t_R$ = 92.5 min) was obtained from subfraction $C_{1-3-1-2}$ (1.3 g) using a preparative HPLC eluted with MeCN–$H_2O$ (25 : 75). Compounds **4** (25 mg; flow rate: 6 mL/min; $t_R$ = 73.2 min) and **8** (17 mg; flow rate: 6 mL/min; $t_R$ = 28.5 min) were isolated from subfraction $C_{1-3-1-5}$ (0.5 g) using a preparative HPLC eluted with MeCN–$H_2O$ (33 : 67). Compounds **1** (19 mg; flow rate: 6 mL/min; $t_R$ = 32.3 min) and **2** (12 mg; flow rate: 6 mL/min; $t_R$ = 37.2 min) were acquired by preparative HPLC eluted with MeCN–$H_2O$ (30 : 70) from subfraction $C_{1-3-1-6}$ (0.2 g). The purities of these compounds ranged from 97.6 to 99.8% as determined by HPLC.

## Identification of isolated compounds

*3β-O-{β-D-xylopyranosyl-(1 → 4)-[β-D-xylopyranosyl-(1 → 2)]-β-D-glucopyranosyl}-2α-hydroxyolean-12-en-28-O-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 2)-α-L-rhamnopyranosyl] ester* (**1**): White powder; m. p. 248–249 °C; $[\alpha]_D^{20}$ + 19.9 (*c* 0.13, MeOH); UV (MeOH): $\lambda_{max}$ (log $\varepsilon$) = 204 (3.96) nm; IR: $\nu_{max}$ = 3375, 2930, 1724, 1641, 1455, 1431, 1369, 1160, 1075, 1044, 972, 921, 897, 825, 627 cm$^{-1}$; $^1$H NMR (500 MHz, $CD_3OD$) data of the aglycon $\delta$ 0.74 (3H, s, $CH_3$-26), 0.84 (3H, s, $CH_3$-24), 0.86 (3H, s, $CH_3$-29), 0.91 (3H, s, $CH_3$-30), 0.96 (3H, s, $CH_3$-25), 1.05 (3H, s, $CH_3$-23), 1.11 (3H, s, $CH_3$-27), 2.86 (1H, dd, *J* = 14.0, 3.0 Hz, H-18), 2.91 (1H, d, *J* = 9.0 Hz, H-3), 3.68 (1H, ddd, *J* = 12.0, 9.0, 3.0 Hz, H-2), 5.30 (1H, br s, H-12); $^{13}$C NMR (125 MHz, $CD_3OD$) data of the aglycon $\delta$ 17.2 (25-$CH_3$), 17.5 (24-$CH_3$), 18.3 (26-$CH_3$), 19.3 (6-$CH_2$), 23.8 (16-$CH_2$), 24.0 (30-$CH_3$), 24.6 (11-$CH_2$), 26.4 (27-$CH_3$), 28.5 (23-$CH_3$), 28.5 (15-$CH_2$), 31.6 (20-C), 33.4 (29-$CH_3$), 33.8 (22-$CH_2$), 33.9 (7-$CH_2$), 34.6 (21-$CH_2$), 37.7 (10-C), 40.7 (8-C), 41.7 (4-C), 42.9 (18-CH), 43.0 (14-C), 46.8 (19-$CH_2$), 47.4 (1-$CH_2$), 48.6 (17-C), 48.8 (9-CH), 56.7 (5-CH), 67.9 (2-CH), 96.5 (3-CH), 124.3 (12-CH), 144.8 (13-C), 177.3 (28-CO); $^1$H NMR (500 MHz, $CD_3OD$) data and $^{13}$C NMR (125 MHz, $CD_3OD$) data for sugar moieties see **Table 1**; HR-ESIMS: *m/z* = 1391.6459 [M + Na]$^+$ (calcd. for $C_{64}H_{104}NaO_{31}$, 1391.6453); ESIMS (positive-ion mode): *m/z* = 1391 [M + Na]$^+$; ESIMS (negative-ion mode): *m/z* = 1367 [M – H]$^-$; ESIMS-MS (positive-ion mode) MS$^2$: *m/z* = 921 [M + Na – 162 – 162 – 146]$^+$, MS$^3$: *m/z* = 789 [M + Na – 162 – 162 – 146 – 132]$^+$, 657 [M + Na – 162 – 162 – 146 – 132 – 132]$^+$; ESIMS-MS (negative-ion mode) MS$^2$: *m/z* = 897 [M – H – 162 – 162 – 146]$^-$, MS$^3$: *m/z* = 765 [M – H – 162 – 162 – 146 – 132]$^-$, MS$^4$: *m/z* = 633 [M – H – 162 – 162 – 146 – 132 – 132]$^-$, MS$^5$: *m/z* = 471 [M – H – 162 – 162 – 146 – 132 – 132 – 162]$^-$.

*3β-O-{β-D-xylopyranosyl-(1 → 4)-[β-D-xylopyranosyl-(1 → 2)]-β-D-xylopyranosyl}-2α-hydroxyolean-12-en-28-O-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 2)-α-L-rhamnopyranosyl] ester* (**2**): White powder; m. p. 230–231 °C; $[\alpha]_D^{20}$ + 22.7 (*c* 0.13, MeOH); UV (MeOH): $\lambda_{max}$ (log $\varepsilon$) = 204 (3.83) nm; IR: $\nu_{max}$ = 3390, 2931, 1724, 1642, 1457, 1430, 1368, 1162, 1049, 972, 921, 896, 825, 625 cm$^{-1}$; $^1$H NMR (500 MHz, $CD_3OD$) data of the aglycon δ 0.74 (3H, s, $CH_3$-26), 0.84 (3H, s, $CH_3$-24), 0.86 (3H, s, $CH_3$-29), 0.91 (3H, s, $CH_3$-30), 0.95 (3H, s, $CH_3$-25), 1.04 (3H, s, $CH_3$-23), 1.11 (3H, s, $CH_3$-27), 2.86 (1H, dd, *J* = 14.0, 3.0 Hz, H-18), 2.89 (1H, d, *J* = 9.0 Hz, H-3), 3.64 (1H, ddd, *J* = 12.0, 9.0, 3.0 Hz, H-2), 5.30 (1H, br s, H-12); $^{13}$C NMR (125 MHz, $CD_3OD$) data of the aglycon δ 17.2 (25-$CH_3$), 17.4 (24-$CH_3$), 18.3 (26-$CH_3$), 19.3 (6-$CH_2$), 23.8 (16-$CH_2$), 24.0 (30-$CH_3$), 24.6 (11-$CH_2$), 26.4 (27-$CH_3$), 28.4 (23-$CH_3$), 28.6 (15-$CH_2$), 31.6 (20-C), 33.4 (29-$CH_3$), 33.8 (22-$CH_2$), 33.9 (7-$CH_2$), 34.6 (21-$CH_2$), 38.8 (10-C), 40.7 (8-C), 41.7 (4-C), 42.9 (18-CH), 43.0 (14-C), 46.8 (19-$CH_2$), 47.5 (1-$CH_2$), 48.6 (17-C), 48.8 (9-CH), 56.7 (5-CH), 67.7 (2-CH), 96.2 (3-CH), 124.3 (12-CH), 144.9 (13-C), 177.3 (28-CO); $^1$H NMR (500 MHz, $CD_3OD$) data and $^{13}$C NMR (125 MHz, $CD_3OD$) data for sugar moieties see **Table 1**; HR-ESIMS: *m/z* = 1361.6344 [M + Na]$^+$ (calcd. for $C_{63}H_{102}NaO_{30}$, 1361.6348); ESIMS (positive-ion mode) m/z: 1361 [M + Na]$^+$; ESIMS (negative-ion mode) *m/z*: 1337 [M – H]$^-$; ESIMS-MS (positive-ion mode) MS$^2$ *m/z*: 891 [M + Na – 162 – 162 – 146]$^+$, MS$^3$: *m/z* = 759 [M + Na – 162 – 162 – 146 – 132]$^+$; ESIMS-MS (negative-ion mode) MS$^2$: *m/z* = 867 [M – H – 162 – 162 – 146]$^+$, MS$^3$: *m/z* = 735 [M – H – 162 – 162 – 146 – 132]$^-$, MS$^4$: *m/z* = 603 [M – H – 162 – 162 – 146 – 132 – 132]$^-$, MS$^5$: *m/z* = 471 [M – H – 162 – 162 – 146 – 132 – 132 – 132]$^-$.

| Position | 1 | | Position | 2 | |
|---|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | | $\delta_H$ | $\delta_C$ |
| 3-O-sugar moieties | | | 3-O-sugar moieties | | |
| D-Glc 1 | 4.40 d (8.0) | 104.8 | D-Xyl 1 | 4.32 d (7.5) | 105.2 |
| 2 | 3.58 dd (9.0, 8.0) | 81.1 | 2 | 3.53 dd (8.0, 7.5) | 77.9 |
| 3 | 3.68 t (9.0) | 76.7 | 3 | 3.63 dd (9.0, 8.0) | 76.5 |
| 4 | 3.56 t (9.0) | 80.0 | 4 | 3.67 m | 77.6 |
| 5 | 3.42 m | 76.0 | 5 | 4.00 dd (10.5, 4.5) | 64.6 |
| 6 | 3.79 m | 61.3 | | 3.29 dd (10.5, 10.0) | |
| | 3.66 m | | | | |
| D-Xyl(1 → 2) 1 | 4.60 d (7.5) | 105.5 | D-Xyl(1 → 2) 1 | 4.56 d (7.5) | 105.8 |
| 2 | 3.14 dd (9.0, 7.5) | 74.8 | 2 | 3.15 dd (8.5, 7.5) | 74.2 |
| 3 | 3.24 t (9.0) | 77.8 | 3 | 3.25 t (9.0) | 77.9 |
| 4 | 3.36 m | 71.5 | 4 | 3.37 m | 71.5 |
| 5 | 3.74 brd (11.5) | 67.0 | 5 | 3.74 dd (11.0, 5.5) | 67.0 |
| | 3.06 dd (11.0, 10.5) | | | 3.07 t (11.0) | |
| D-Xyl(1 → 4) 1 | 4.28 d (8.0) | 105.3 | D-Xyl(1 → 4) 1 | 4.26 d (7.5) | 103.9 |
| 2 | 3.13 t (8.5) | 75.0 | 2 | 3.17 dd (9.0, 7.5) | 75.1 |
| 3 | 3.25 dd (9.0, 9.5) | 77.8 | 3 | 3.31 dd (9.0, 8.5) | 77.6 |
| 4 | 3.45 m | 70.9 | 4 | 3.44 m | 71.0 |
| 5 | 3.84 dd (11.5, 4.5) | 67.1 | 5 | 3.82 dd (10.0, 5.0) | 67.0 |
| | 3.17 dd (12.0, 9.5) | | | 3.18 m | |
| 28-O-sugar moieties | | | 28-O-sugar moieties | | |
| L-Rha 1 | 6.29 br s | 94.0 | L-Rha 1 | 6.29 br s | 94.0 |
| 2 | 3.75 br s | 81.6 | 2 | 3.70 br s | 81.6 |
| 3 | 3.62 m | 72.2 | 3 | 3.63 m | 72.2 |
| 4 | 3.37 m | 73.7 | 4 | 3.39 m | 73.7 |
| 5 | 3.68 m | 72.3 | 5 | 3.67 m | 72.3 |
| 6 | 1.17 d (6.0) | 18.1 | 6 | 1.17 d (6.0) | 18.1 |
| D-Glc(1 → 2) 1 | 4.36 d (7.5) | 106.9 | D-Glc(1 → 2) 1 | 4.36 d (7.5) | 106.9 |
| 2 | 3.23 dd (8.0, 7.5) | 76.3 | 2 | 3.24 t (8.0) | 76.1 |
| 3 | 3.29 t (8.0) | 77.9 | 3 | 3.29 t (8.0) | 77.7 |
| 4 | 3.35 m | 71.2 | 4 | 3.34 m | 71.2 |
| 5 | 3.30 m | 76.9 | 5 | 3.32 m | 76.9 |
| 6 | 4.06 brd (9.5) | 69.9 | 6 | 4.06 brd (10.0) | 70.0 |
| | 3.70 brd (11.5) | | | 3.72 brd (11.5) | |
| D-Glc(1 → 6) 1 | 4.24 d (8.0) | 104.7 | D-Glc(1 → 6) 1 | 4.25 d (8.0) | 104.8 |
| 2 | 3.17 t (8.5) | 75.2 | 2 | 3.17 t (8.5) | 75.2 |
| 3 | 3.18 t (8.5) | 77.9 | 3 | 3.18 t (8.5) | 77.8 |
| 4 | 3.23 t (9.0) | 71.2 | 4 | 3.24 t (9.0) | 71.2 |
| 5 | 3.30 m | 77.7 | 5 | 3.31 m | 77.9 |
| 6 | 3.81 brd (10.5) | 62.7 | 6 | 3.81 brd (10.5) | 62.8 |
| | 3.60 brd (12.0) | | | 3.60 brd (12.0) | |

**Table 1** $^1$H and $^{13}$C spectroscopic NMR data for sugar moieties of compounds **1**–**2** (500 MHz for $^1$H NMR and 125 MHz for $^{13}$C NMR, $CD_3OD$)[a].

[a] *J* values are in parentheses and reported in Hz; chemical shifts are given in ppm; assignments were confirmed by 1D-TOCSY, HSQC, and HMBC experiments

*Methyl 3β-hydroxy-erythrosuamate* (**3**): White powder; m.p. 111–112 °C; $[\alpha]_D^{20}$ − 92.4 (*c* 0.08, EtOH); UV (EtOH): $\lambda_{max}$ (log $\varepsilon$) = 223 (4.24) nm; IR: $\nu_{max}$ = 3459, 2945, 2882, 1717, 1646, 1435, 1391, 1259, 1190, 1158, 967, 939, 871 cm$^{-1}$; $^1$H NMR (500 MHz, $CDCl_3$) data, see **Table 2**; $^{13}$C NMR (125 MHz, $CDCl_3$) data, see **Table 3**; HR-ESIMS: *m/z* = 431.2054 $[M + Na]^+$ (calcd. for $C_{22}H_{32}NaO_7$, 431.2040); ESIMS (positive-ion mode): *m/z* = 409 $[M + H]^+$, 431 $[M + Na]^+$, 447 $[M + K]^+$; ESIMS (negative-ion mode): *m/z* = 407 $[M - H]^-$, 443 $[M + Cl]^-$.

*Methyl 3β-acetoxy-erythrosuamate* (**4**): White powder; m.p. 96–97 °C; $[\alpha]_D^{20}$ − 82.2 (*c* 0.08, EtOH); UV (EtOH): $\lambda_{max}$ (log $\varepsilon$) = 223 (4.19) nm; IR: $\nu_{max}$ = 3473, 2947, 1721, 1649, 1435, 1375, 1261, 1238, 1158, 1033, 968, 871 cm$^{-1}$; $^1$H NMR (500 MHz, $CDCl_3$) data, see **Table 2**; $^{13}$C NMR (125 MHz, $CDCl_3$) data, see **Table 3**; HR-ESIMS: *m/z* = 473.2167 $[M + Na]^+$ (calcd. for $C_{24}H_{34}NaO_8$, 473.2145); ESIMS (positive-ion mode): *m/z* = 451 $[M + H]^+$, 473 $[M + Na]^+$, 489 $[M + K]^+$; ESIMS (negative-ion mode): *m/z* = 449 $[M - H]^-$, 485 $[M + Cl]^-$.

*Ethyl 3β-hydroxy-erythrosuamate* (**5**): White powder; m.p. 84–85 °C; $[\alpha]_D^{20}$ − 82.8 (*c* 0.08, EtOH); UV (EtOH): $\lambda_{max}$ (log $\varepsilon$) = 223 (4.23) nm; IR: $\nu_{max}$ = 3470, 2973, 2944, 1715, 1647, 1458, 1394, 1259, 1198, 1157, 1036, 967, 873 cm$^{-1}$; $^1$H NMR (500 MHz, $CDCl_3$) data, see **Table 2**; $^{13}$C NMR (125 MHz, $CDCl_3$) data, see **Table 3**; HR-ESIMS: *m/z* = 423.2392 $[M + H]^+$ (calcd. for $C_{23}H_{35}O_7$, 423.2377); ESIMS (positive-ion mode): *m/z* = 423 $[M + H]^+$, 445 $[M + Na]^+$, 461 $[M + K]^+$; ESIMS (negative-ion mode): *m/z* = 421 $[M - H]^-$, 458 $[M + Cl]^-$.

*Ethyl 3β-acetoxy-erythrosuamate* (**6**): White powder; m.p. 85–86 °C; $[\alpha]_D^{20}$ − 88.0 (*c* 0.08, EtOH); UV (EtOH): $\lambda_{max}$ (log $\varepsilon$) = 223 (4.26) nm; IR: $\nu_{max}$ = 3479, 2973, 2945, 1731, 1715, 1646, 1457, 1375, 1260, 1237, 1155, 1032, 968, 871 cm$^{-1}$; $^1$H NMR (500 MHz, $CDCl_3$) data, see **Table 2**; $^{13}$C NMR (125 MHz, $CDCl_3$) data, see **Table 3**; HR-ESIMS *m/z*: 465.2505 $[M + H]^+$ (calcd. for $C_{25}H_{37}O_8$, 465.2482); ESIMS (positive-ion mode) *m/z*: 465 $[M + H]^+$, 487 $[M + Na]^+$, 503 $[M + K]^+$; ESIMS (negative-ion mode) *m/z*: 463 $[M - H]^-$, 499 $[M + Cl]^-$.

**Table 2** $^1$H NMR spectroscopic data for compounds **3–7** (500 MHz, $CDCl_3$) and compound **8** (500 MHz, $CD_3OD$)[a].

| Position | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|
| 1 | 1.89 m, 1.36 m | 1.91 m, 1.43 m | 1.89 m, 1.34 m | 1.87 m, 1.41 m | 1.88 m, 1.22 m | 1.81 m, 1.09 m |
| 2 | 2.14 m, 1.76 m | 2.26 m, 1.74 m | 2.14 m, 1.76 m | 2.23 m, 1.73 m | 1.90 m, 1.23 m | 1.65 m, 1.44 m |
| 3 | 3.35 m | 4.67 dd (12.0, 4.5) | 3.35 m | 4.66 dd (12.0, 4.5) | 3.10 dt (12.0, 4.0) | 2.15 m, 1.10 m |
| 5 | 2.33 s | 2.44 s | 2.33 s | 2.43 s | 1.36 d (12.0) | 1.40 d (11.0) |
| 6 | | | | | 4.60 d (12.0) | 4.71 d (11.0) |
| 7 | 3.92 d (10.5) | 3.95 d (10.5) | 3.92 d (10.5) | 3.94 d (10.0) | | |
| 8 | 1.85 m | 1.86 m | 1.85 m | 1.85 m | 2.39 dd (12.5, 1.5) | 2.45 dd (13.5, 2.5) |
| 9 | 1.68 dt (12.0, 3.0) | 1.73 m | 1.68 dt (12.0, 3.5) | 1.69 m | 1.67 m | 1.74 dt (13.0, 3.5) |
| 11 | 2.08 m, 1.18 m | 1.93 m, 1.21 m | 1.92 m, 1.21 m | 1.89 m, 1.18 m | 2.01 m, 1.23 m | 1.98 m, 1.18 m |
| 12 | 3.80 m, 2.04 m | 3.83 m, 2.05 m | 3.81 m, 2.01 m | 3.81 m, 2.00 m | 3.79 m, 2.05 m | 3.71 m, 2.03 m |
| 14 | 2.81 m | 2.83 m | 2.81 m | 2.81 m | 2.97 m | 2.93 m |
| 15 | 5.73 br s | 5.76 br s | 5.73 br s | 5.73 br s | 5.70 br s | 5.73 br s |
| 17 | 1.19 d (7.0) | 1.21 d (7.0) | 1.18 d (6.5) | 1.20 d (7.0) | 1.13 d (7.0) | 1.09 d (7.0) |
| 18 | 1.36 s | 1.21 s | 1.36 s | 1.20 s | 1.70 s | 1.36 s |
| 20 | 0.90 s | 0.99 s | 0.90 s | 0.98 s | 0.89 s | 0.84 s |
| 21 | 3.74 s | 3.78 s | 3.75 s | 3.77 s | 3.75 s | 3.63 s |
| 22 | 3.68 s | 3.70 s | 4.13 q (7.0) | 4.14 q (7.0) | 4.14 q (7.0) | |
| 23 | | | 1.27 t (7.0) | 1.27 t (7.0) | 1.27 t (7.0) | |
| 1′ | | | | | | 5.52 d (8.0) |
| 2′ | | 2.05 s | | 2.03 s | | 3.29 m |
| 3′ | | | | | | 3.37 m |
| 4′ | | | | | | 3.32 m |
| 5′ | | | | | | 3.34 m |
| 6′ | | | | | | 3.77 dd (11.5, 3.5) |
| | | | | | | 3.59 dd (12, 5) |

[a] *J* values are in parentheses and reported in Hz; chemical shifts are given in ppm; assignments were confirmed by HSQC and HMBC experiments

**Table 3** $^{13}$C NMR spectroscopic data for compounds **3–7** (125 MHz, $CDCl_3$) and compound **8** (125 MHz, $CD_3OD$).

| Position | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|
| 1 | 37.1 | 36.5 | 37.1 | 36.5 | 37.9 | 40.7 |
| 2 | 27.5 | 23.5 | 27.6 | 23.4 | 27.8 | 20.3 |
| 3 | 78.0 | 78.7 | 78.1 | 78.7 | 78.0 | 40.7 |
| 4 | 47.6 | 45.7 | 47.7 | 45.7 | 50.3 | 46.4 |
| 5 | 64.3 | 64.6 | 64.3 | 64.6 | 58.1 | 59.7 |
| 6 | 207.9 | 207.9 | 207.9 | 208.0 | 75.4 | 76.9 |
| 7 | 75.7 | 75.7 | 75.8 | 75.7 | 209.9 | 211.2 |
| 8 | 50.8 | 50.9 | 50.8 | 51.2 | 51.0 | 52.8 |
| 9 | 46.0 | 46.4 | 46.1 | 46.4 | 46.3 | 47.9 |
| 10 | 43.1 | 43.0 | 43.1 | 43.0 | 37.6 | 38.9 |
| 11 | 26.2 | 26.2 | 26.2 | 26.2 | 27.3 | 28.4 |
| 12 | 23.5 | 23.7 | 23.5 | 23.7 | 23.5 | 25.1 |
| 13 | 165.1 | 164.9 | 164.7 | 164.5 | 163.9 | 169.3 |
| 14 | 40.2 | 40.2 | 40.2 | 40.2 | 39.4 | 41.1 |
| 15 | 113.2 | 113.4 | 113.7 | 113.8 | 113.5 | 113.4 |
| 16 | 167.2 | 167.2 | 166.8 | 166.8 | 166.7 | 166.5 |
| 17 | 13.7 | 13.7 | 13.7 | 13.7 | 14.8 | 15.4 |
| 18 | 25.5 | 25.7 | 25.5 | 25.7 | 25.3 | 32.1 |
| 19 | 174.1 | 172.6 | 174.1 | 172.6 | 178.0 | 179.0 |
| 20 | 14.4 | 14.4 | 14.4 | 14.3 | 13.5 | 14.2 |
| 21 | 51.8 | 51.8 | 51.8 | 51.2 | 51.7 | 52.0 |
| 22 | 50.9 | 51.2 | 59.6 | 59.6 | 59.7 | |
| 23 | | | 14.2 | 14.2 | 14.2 | |
| 1′ | | 170.4 | | 170.4 | | 95.2 |
| 2′ | | 21.0 | | 21.0 | | 73.9 |
| 3′ | | | | | | 78.0 |
| 4′ | | | | | | 71.1 |
| 5′ | | | | | | 78.7 |
| 6′ | | | | | | 62.3 |

[a] Chemical shifts are given in ppm; assignments were confirmed by HSQC and HMBC experiments

*Ethyl 3β,6α-dihydroxy-cassamate* (**7**): White powder; m.p. 69–70 °C; $[\alpha]_D^{20}$ – 36.6 (*c* 0.08, EtOH); UV (EtOH): $\lambda_{max}$ (log $\varepsilon$) = 223 (4.18) nm; IR: $\nu_{max}$ = 3453, 2943, 2878, 1072, 1648, 1455, 1391, 1154, 1096, 980, 868 cm$^{-1}$; $^1$H NMR (500 MHz, $CDCl_3$) data, see **Table 2**; $^{13}$C NMR (125 MHz, $CDCl_3$) data, see **Table 3**; HR-ESIMS: *m/z* = 445.2212 $[M + Na]^+$ (calcd. for $C_{23}H_{34}NaO_7$, 445.2196); ESIMS (positive-ion mode): *m/z* = 445 $[M + Na]^+$, 461 $[M + K]^+$; ESIMS (negative-ion mode): *m/z* = 421 $[M - H]^-$, 457 $[M + Cl]^-$.

*β-D-glucopyranosyl 6α-hydroxy-cassamate* (**8**): White powder; m.p. 146–147 °C; $[\alpha]_D^{20}$ + 43.6 (*c* 0.07, EtOH); UV (EtOH): $\lambda_{max}$ (log $\varepsilon$) = 223 (4.22) nm; IR: $\nu_{max}$ = 3459, 2945, 2879, 1716, 1643, 1458, 1155, 1072, 952 cm$^{-1}$; $^1$H NMR (500 MHz, $CD_3OH$) data, see **Table 2**; $^{13}$C NMR (125 MHz, $CD_3OH$) data, see **Table 3**; HR-ESIMS: *m/z* = 563.2467 $[M + Na]^+$ (calcd. for $C_{27}H_{40}NaO_{11}$, 563.2463); ESIMS (positive-ion mode): *m/z* = 563 $[M + Na]^+$, 579 $[M + K]^+$; ESIMS (negative-ion mode): *m/z* = 539 $[M - H]^-$, 575 $[M + Cl]^-$.

## Acid hydrolysis and determination of the absolute configuration of the monosaccharides of compounds 1 and 2

Compounds **1** (3 mg) and **2** (3 mg) were hydrolyzed separately with 2 M HCl for 10 h at 95 °C. The reaction mixture was extracted with EtOAc; the aqueous layer was evaporated under a vacuum and diluted repeatedly with $H_2O$ and evaporated *in vacuo* to furnish a neutral residue. The residue was dissolved in anhydrous pyridine (1 mL), to which 2 mg L-cysteine methyl ester hydrochloride were added. The mixture was stirred at 60 °C for 2 h, and, after evaporation *in vacuo* to dryness, 0.2 mL *N*-trimethylsilylimidazole were added and kept at 60 °C for another 2 h [12,13]. The reaction mixture was partitioned between *n*-hexane and $H_2O$ (2 mL each) and the *n*-hexane extract analyzed

by comparing the retention times of derivatives of sugars obtained from the water layer of the hydrolysis solution with those of standard samples using GC, which was performed under the following conditions: capillary column, HP-5 (30 m × 0.25 mm, with a 0.25 µm film; Dikma); detection, FID; detector temperature, 280 °C; injection temperature, 250 °C; initial temperature 160 °C, then raised to 280 °C at 5 °C/min, final temperature was maintained for 10 min; carrier, He gas. Peaks were observed with $t_R$ (min) of 17.8 (D-xylose), 18.5 (L-rhamnose), and 19.6 (D-glucose). D-xylose, L-rhamnose, and D-glucose were obtained in the ratio 2 : 1 : 3 and 3 : 1 : 2 from **1** and **2**, respectively.

### Acid hydrolysis of 8

The hydrolysis and derivation method of the residue and the GC analysis were the same as those described above. Peaks were observed with $t_R$ (min) of 19.6 (D-glucose).

### Cytotoxicity assays

The cytotoxicity assay against HCT-8 (human colon cancer), Bel-7402 (human hepatoma cancer), BGC-823 (human gastric cancer), A549 (human lung epithelial), and A2780 (human ovarian cancer) cells ($IC_{50}$) was assessed using the MTT method as described in the literature [14]. Camptothecin (Sigma; 95% purity; HPLC grade) was used as the positive control.

### Supporting information

Original spectra for compounds **1**–**8** (**Figs. 1** and **2**) are available as Supporting Information.

## Results and Discussion

Compound **1** exhibited a pseudomolecular ion at $m/z$ = 1391.6459 $[M + Na]^+$ in the HR-ESIMS, consistent with the molecular formula $C_{64}H_{104}O_{31}$. The $^{13}C$ NMR spectrum of **1** showed 64 signals, of which 30 were assigned to a triterpenoid moiety and 34 to the saccharide portion. The seven tertiary methyl protons ($\delta_H$ = 0.74, 0.84, 0.86, 0.91, 0.96, 1.05, and 1.11) and a characteristic olefinic proton [$\delta_H$ = 5.30 (1H, br s)] in the $^1H$ NMR spectrum coupled with information from the $^{13}C$ NMR spectrum, seven methyl carbons ($\delta_C$ = 17.2, 17.5, 18.3, 24.0, 26.4, 28.5, and 33.4) and a pair of olefinic carbons ($\delta_C$ = 124.3 and 144.8) indicated that the aglycon possesses an olean-12-ene skeleton (see Materials and Methods section). Further features were signals at $\delta_H$ = 3.68 (1H, ddd, $J$ = 12.0, 9.0, 3.0 Hz, H-2) and 2.91 (1H, d, $J$ = 9.0 Hz, H-3), indicative of secondary alcoholic functions. Thus, the aglycon of **1** was identified as 2α,3β-dihydroxy-olean-12-en-28-oic acid (maslinic acid) by comparison of $^1H$ and $^{13}C$ NMR spectroscopic data of **1** obtained from the 2D NMR spectrum with those reported in the literature [15]. Glycosidation of the alcohol group function at C-3 was indicated by the downshift (~ 12 ppm), and the carboxyl at C-28 was indicated by the upshift (~ 2 ppm), observed for these carbon resonances in **1**, if compared to the corresponding signals in non-glycosidated model compounds [16].

For the sugar portion, the $^1H$ NMR spectrum (**Table 1**) of **1** displayed signals for six anomeric proton signals at $\delta_H$ = 4.24 (d, $J$ = 8.0 Hz), 4.28 (d, $J$ = 8.0 Hz), 4.36 (d, $J$ = 7.5 Hz), 4.40 (d, $J$ = 8.0 Hz), 4.60 (d, $J$ = 7.5 Hz), and 6.29 (br s) and one methyl doublet $\delta_H$ = 1.17 (d, $J$ = 6.0 Hz), which gave HSQC correlations with six anomeric carbon signals at $\delta_C$ = 104.7, 105.3, 106.9, 104.8, 105.5, and 94.0 and one methyl carbon at $\delta_C$ = 18.1, respectively, suggesting the occurrence of six monosaccharide units including one deoxyhexose unit. The chemical shifts of all the individual protons of the six sugar units were attributed on the basis of 1D TOCSY spectral analysis, and the $^{13}C$ chemical shifts of their relative attached carbons were clearly assigned from the HSQC spectrum (**Table 1**). These data showed the presence of three β-glucopyranoses (Glc, Glc I, Glc II), two β-xylopyranoses (Xyl I, Xyl II), and one α-rhamnopyranose (Rha). The configurations of the sugar units were determined to be D for glucose and xylose and L for rhamnose by GC analysis of trimethylsilylated derivatives of the sugars in the hydrolysate of **1** (see Materials and Methods section) [12, 13]. The sugar sequences of **1** were established by ESIMS-MS in the negative ion mode. The ESIMS of **1** exhibited a deprotonated molecular ion peak $[M - H]^-$ at $m/z$ = 1367. The abundance of the diagnostic fragment ion peak at $m/z$ = 897 $[M - H - 162 - 162 - 146]^-$ in $ESIMS^2$ suggested that the first sugar chain consists of two glucopyranosyls, and one rhamnopyranosyl

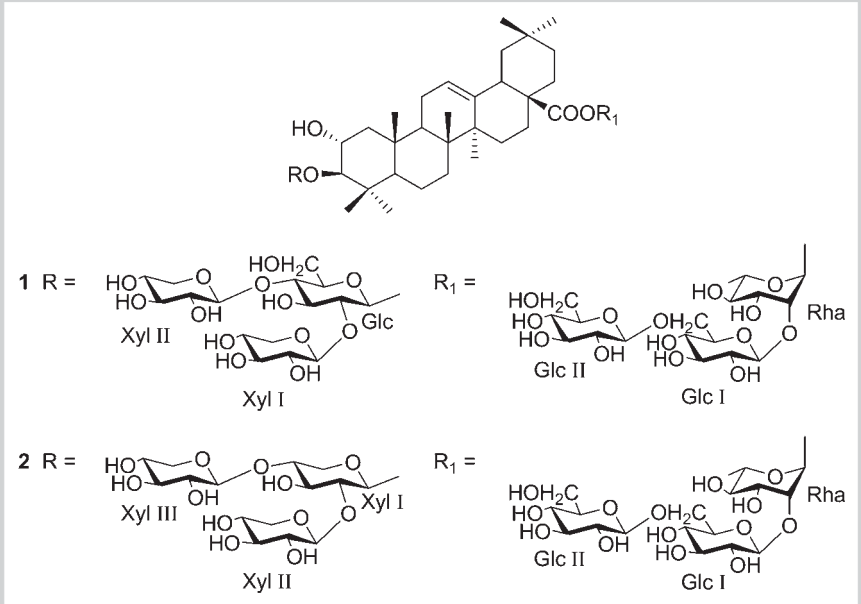


**Fig. 1** Chemical structures of compounds **1** and **2**.

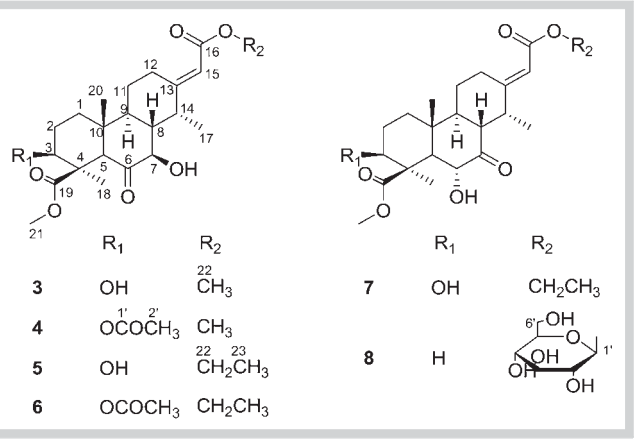


**Fig. 2** Chemical structures of compounds **3**–**8**.

is ester linked to the aglycon [17]. Further fragments at *m/z* = 765 [M – H – 162 – 162 – 146 – 132]$^-$, 633 [M – H – 162 – 162 – 146 – 132 – 132]$^-$, and 471 [M – H – 162 – 162 – 146 – 132 – 132 – 162]$^-$ in ESIMS$^n$, corresponding to the elimination of two xylopyranosyls and one glucopyranosyl, allowed us to establish the glucopyranose as inner sugar for the latter chain. The location of the trisaccharide chains at C-3 and C-28 were unambiguously defined by the HMBC experiment, which showed long-range correlations between H-1 ($\delta$ 4.40) of Glc and C-3 ($\delta$ 96.5) of the aglycon, H-1 ($\delta$ 4.60) of Xyl I and C-2 ($\delta$ 81.1) of Glc, H-1 ($\delta$ 4.28) of Xyl II and C-4 ($\delta$ 80.0) of Glc, H-1 ($\delta$ 6.29) of Rha and C-28 ($\delta$ 177.3) of the aglycon, H-1 ($\delta$ 4.36) of Glc I and C-2 ($\delta$ 81.6) of Rha, H-1 ($\delta$ 4.24) of Glc II, and C-6 ($\delta$ 69.9) of Glc I, as shown in **Fig. 3**. Hence, on the basis of this evidence, the structure of **1** was proposed as 3*β*-*O*-{*β*-D-xylopyranosyl-(1 → 4)-[*β*-D-xylopyranosyl-(1 → 2)]-*β*-D-glucopyranosyl}-2*α*-hydroxyolean-12-en-28-*O*-[*β*-D-glucopyranosyl-(1 → 6)-*β*-D-glucopyranosyl-(1 → 2)-*α*-L-rhamnopyranosyl] ester (**Fig. 1**).

Compound **2** exhibited a pseudomolecular ion peak at *m/z* 1361.6344 [M + Na]$^+$ in the HR-ESIMS, ascribable to the molecular formula $C_{63}H_{102}O_{30}$. The $^1$H and $^{13}$C NMR signals of **2** assigned from the 2D NMR spectra were almost superimposable on those of **1** except for the characteristic signals of a xylopyranosyl moiety instead of a glucopyranosyl moiety linked at C-3. Accordingly, the ESIMS experiment (negative-ion mode) gave a quasi-molecular ion peak [M – H]$^-$ at *m/z* = 1337 indicating a molecular weight of 1338 for compound **2**. Further diagnostic fragment ion peaks in the ESIMS-MS spectrum were observed at *m/z* = 867 [M – H – 162 – 162 – 146]$^-$, 735 [M – H – 162 – 162 – 146 – 132]$^-$, 603 [M – H – 162 – 162 – 146 – 132 – 132]$^-$ and 471 [M – H – 162 – 162 – 146 – 132 – 132 – 132]$^-$ corresponding to the successive loss of three xylopyranosyls. In particular, the HMBC correlations between H-1 ($\delta$ 4.32) of Xyl I and C-3 ($\delta$ 96.2) of the aglycon, H-1 ($\delta$ 4.56) of Xyl II and C-2 ($\delta$ 77.9) of Xyl I, and between H-1 ($\delta$ 4.26) of Xyl III and C-4 ($\delta$ 77.6) of Xyl I supported the linkage sites of the sugar chain attached to C-3. Moreover, the configuration of the sugar units were determined as reported for compound **1**. Hence, the structure of **2** was established as 3*β*-*O*-{*β*-D-xylopyranosyl-(1 → 4)-[*β*-D-xylopyranosyl-(1 → 2)]-*β*-D-xylopyranosyl}-2*α*-hydroxyolean-12-en-28-*O*-[*β*-D-glucopyranosyl-(1 → 6)-*β*-D-glucopyranosyl-(1 → 2)-*α*-L-rhamnopyranosyl] ester.

Compound **3** showed the molecular formula of $C_{22}H_{32}O_7$ deduced from its HR-ESIMS (*m/z* = 431.2054) [M + Na]$^+$, which agreed with methyl 3*β*-hydroxy-erythrosuamate. Because just few $^1$H and $^{13}$C NMR data of compound **3** were reported in the literature [18], detailed $^1$H and $^{13}$C NMR data were also described and assigned in this paper (**Tables 2** and **3**). In the IR spectrum, bands due to hydroxyl (3459 cm$^{-1}$) and carbonyl (1717 cm$^{-1}$) groups were clearly seen. The $^1$H NMR spectrum showed characteristic signals of cassaine-type diterpenoid, i.e., three methyls and two methoxy groups [$\delta_H$ = 1.19 (3H, d, *J* = 7.0 Hz, H-17), 1.36, 0.90, 3.74, 3.68 (each 3H, s, H-18, 20, 21, 22)] and an olefinic proton [$\delta_H$ = 5.73 (1H, brs, H-15)]. Its $^{13}$C NMR and DEPT spectra revealed a pair of olefinic carbons ($\delta_C$ = 113.2, 165.1) and another three carbonyl carbons ($\delta_C$ = 167.2, 174.1, 207.9) [4,10]. Furthermore, evidence for a hydroxy group at C-3*β*, and the 6-keto-7*β*-hydroxy group and the methyl esterified at C-16 were provided by detailed analyses of HSQC, HMBC, and NOE correlations. Therefore, methyl 3*β*-hydroxy-erythrosuamate has structure **3**, as shown in **Fig. 2**.

The molecular formula of **4** was assigned as $C_{24}H_{34}O_8$ based on the [M + Na]$^+$ peak at *m/z* = 473.2167 in the HR-ESIMS. Strong IR bands due to hydroxyl (3473 cm$^{-1}$) and carbonyl groups (1721 cm$^{-1}$) were clearly seen. Its $^1$H NMR spectrum showed signals for one olefinic proton, two oxymethines, four methyls, and two methoxys. Its $^{13}$C NMR and DEPT spectra indicated signals due to six methyls, four methylenes, seven methines, and seven quaternary carbons. Careful analysis of the NMR data indicated that **4** possessed a cassaine-type diterpenoid skeleton closely related to that of **3**. The presence of a 6-keto-7-hydroxy group was confirmed by the important connectivities of H-5 ($\delta_H$ = 2.44) with C-6 ($\delta_C$ = 207.9) and H-7 ($\delta_H$ = 3.95) with C-8 ($\delta_C$ = 50.9) found in the HMBC spectrum. The position of *exo*-*α*,*β*-unsaturated methyl ester moiety connected to C-13, was based on HMBC correlations of H-15 ($\delta_H$ = 5.76) with C-12 ($\delta_C$ = 23.7), C-14 ($\delta_H$ = 40.2) and C-16 ($\delta_C$ = 167.2), and H-22 ($\delta_H$ = 3.70) with C-16 ($\delta_C$ = 167.2). Compared to **3**, two extra resonances at $\delta_C$ = 21.0 and 170.4 assigned to an acetyl group were observed. The HMBC correlations of H-3 ($\delta_H$ = 4.67, dd, *J* = 12.0, 4.5 Hz) with carbonyls C-1′ ($\delta_C$ = 170.4) of

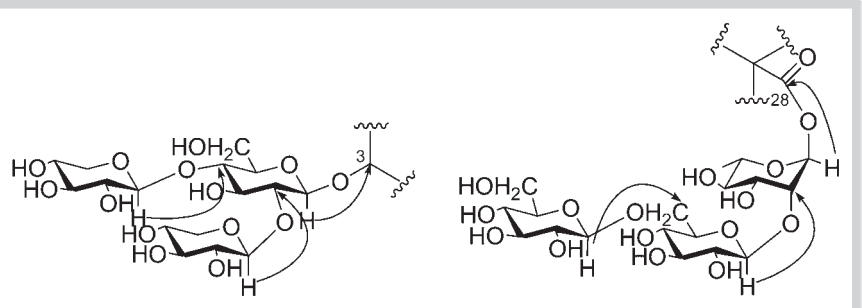

**Fig. 3** Significant HMBC correlations of the sugar chains of **1**.

acetyl established that the acetyl group esterified the hydroxy group at C-3. The relative configuration of **4** was deduced from its NOE spectrum. Specifically, correlations were clearly observed between H-3 ($\delta_H$ = 4.67), H-5 ($\delta_H$ = 2.44) and $H_3$-18 ($\delta_H$ = 1.21), H-7 ($\delta_H$ = 3.95), H-9 ($\delta_H$ = 1.73) and $H_3$-17 ($\delta_H$ = 1.21), $H_3$-20 ($\delta_H$ = 0.99) and H-8 ($\delta_H$ = 1.86), and $H_3$-20 ($\delta_H$ = 0.99) and $H_3$-21 ($\delta_H$ = 3.78), unambiguously indicated that 3α-H, 5α-H, 7α-H, 8β-H, 9α-H, and 18α-H were attached to rings, respectively. Also the NOE correlation between H-14 ($\delta_H$ = 2.83) and H-15 ($\delta_H$ = 5.76) was in favor of the *E* configuration for the double bound at C-13/C-15. Thus, compound **4** was concluded to be methyl 3β-acetoxy-erythrosuamate.

The molecular formula of **5** was established as $C_{23}H_{34}O_7$. Its $^1$H and $^{13}$C NMR data were very similar to those of **3**, except the presence of an ethoxy group and lack of a methoxy group at C-16. The correlations from oxymethylene protons H-22 ($\delta_H$ = 4.13) to carbonyl carbon C-16 ($\delta_C$ = 166.8) and methyl carbon C-23 ($\delta_C$ = 14.2) in its HMBC spectrum confirmed the above deduction. Thus, the structure of **5** was established as ethyl 3β-hydroxy-erythrosuamate.

Compound **6** had the molecular formula of $C_{25}H_{36}O_8$, as deduced from the quasi-molecular ion peak at $m/z$ = 465.2505 $[M + H]^+$ by the HR-ESIMS. The spectroscopic data (**Tables 2** and **3**) of **6** resembled those of **4** except for the replacement of the 16-methoxy group with an ethoxy function. Thus, the structure of compound **6** was assigned as ethyl 3β-acetoxy-erythrosuamate.

Compound **7** was found by HR-ESIMS to possess the molecular formula $C_{23}H_{34}O_7$, the same as **5**. Detailed analysis of the $^1$H and $^{13}$C NMR spectrum of **7** in comparison with those of **5** showed a difference in the replacement of the 6-keto-7β-hydroxy in **5** by the 6α-hydroxy-7-keto in **7**. In particular, the HMBC correlations of H-5 ($\delta_H$ = 1.36) with C-6 ($\delta_C$ = 75.4) and H-6 ($\delta_H$ = 4.60) with C-7 ($\delta_C$ = 209.9) as well as the NOE interaction between H-6 and $H_3$-20 confirmed the occurrence of 6α-hydroxy-7-keto. Accordingly, compound **7** was identified to be ethyl 3β,6α-dihydroxy-cassamate.

The molecular formula of compound **8** was determined to be $C_{27}H_{40}O_{11}$ by HR-ESIMS. Its spectroscopic properties indicated the presence of a sugar unit and a diterpenoid unit analogous to erythrofordin C [10]. In the $^1$H NMR spectrum, one anomeric proton [$\delta_H$ = 5.52 (1H, d, $J$ = 8.0 Hz)] was observed, and in the $^{13}$C NMR spectrum, 6 signals were assigned to an β-glucopyranose. The absolute configuration of the glucose was determined to be in the D-series by the GC method described in the experiment section [12, 13]. Comparison of the $^{13}$C NMR data of the aglycon of **8** with those of erythrofordin C indicated that they shared the same skeleton, being the absence of hydroxy at C-3 the most notable difference, suggested by the disappearance of the great number of signals at $\delta_C$ = 79.1 assigned to the oxymethine group. Assignments of all chemical shifts of protons and carbons of the aglycone portion were ascertained from a combination of HSQC and HMBC analyses. A linkage of the monoglycoside of the β-D-glucopyranosyl to C-16 of the aglycone via an ester bond was proved by a HMBC correlation between the anomeric proton at $\delta_H$ = 5.52 and C-16 of the aglycone at $\delta_C$ = 166.5. Thus, compound **8** is β-D-glucopyranosyl 6α-hydroxy-cassamate.

Selected compounds (**3**–**8**) were evaluated for their cytotoxicity against HCT-8, Bel-7402, BGC-823, A549, and A2780 cell lines. As determined by a MTT assay, the active substances were compounds **5**–**7**, which had an ethoxy attached to C-16 and showed moderate cytotoxic activity against these cell lines (**Table 4**). It is inferred that the length of the alkoxyl chain connected to C-16 in cassaine-type diterpenoids might influence the cytotoxicity.

**Table 4** Cytotoxicity of compounds **5**–**7** against five human cancer cell lines.

| Compound[a] | $IC_{50}$ (μM) | | | | |
|---|---|---|---|---|---|
| | HCT-8 | Bel-7402 | BGC-823 | A549 | A2780 |
| **5** | > 10 | 3.67 | 3.29 | 6.48 | 2.94 |
| **6** | 8.68 | 3.26 | 3.47 | 4.04 | 1.51 |
| **7** | > 10 | 5.46 | 3.49 | 3.10 | 2.49 |
| Camptothecin[b] | 3.20 | 12.51 | 9.72 | 3.11 | 0.29 |

HCT-8 = human colon cancer cell line; Bel-7402 = human hepatoma cell line; BGC-823 = human gastric carcinoma cell line; A549 = human lung epithelial cell line; A2780 = human epithelial carcinoma cell line. " > 10" = inactive. [a] Compounds **3**, **4**, and **8** were inactive ($IC_{50}$ > 10 μM) for all cells. [b] Positive control

This is the first detailed report on the leaves of *Erythrophleum fordii* with respect to its terpenoid and their glycosides. We found two saponins containing oleanlic acid aglycon (**1** and **2**), which, though common in other plants, have not been reported so far from *Erythrophleum* genus. However, the ethyl esterified products, such as **5**–**7**, might be artefacts formed during the extraction process. Compounds **3**–**8** were evaluated for their cytotoxicity against a small panel of cell lines; only **5**–**7** demonstrated moderated cytotoxic activity.

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## References

1 *Chen JS, Zhen S.* Chinese virose plant, 1st edition. Beijing: Beijing Science Press; 1987: 321
2 *Editorial Committee of the Flora of China.* Flora of China, 1st edition. Beijing: Beijing Science Press; 1985: 321
3 *Cui YL, Ran XD.* Zhong Hua Yiao Hai, 1st edition. Harbin: Harbin Press; 1993: 1402
4 *Qu J, Hu YC, Yu SS, Chen XG, Li Y.* New cassaine diterpenoid amides with cytotoxic activities from the bark of *Erythrophleum fordii*. Planta Med 2006; 72: 442–449
5 *Cronlund A.* New alkaloids from *Erythrophleum* species. Planta Med 1973; 24: 371–374
6 *Morin RB.* The alkaloids: chemistry and physiology, Vol. 10. New York: New York Academic Press; 1968: 287–303
7 *Qu J, Yu SS, Tang WZ, Liu YB, Liu Y, Liu J.* Progress on cassaine-type diterpenoid ester amines and amides (*Erythrophleum* Alkaloids). Nat Prod Commun 2006; 1: 839–850
8 *Li N, Yu F, Yu SS.* Triterpenoids from *Erythrophleum fordii*. Acta Bot Sin 2004; 46: 371–374
9 *Yu F, Li N, Yu SS.* A new diterpenoid glucopyranoside from *Erythrophleum fordii*. J Asian Nat Prod Res 2005; 7: 19–24

10 *Tsao CC, Shen YC, Su CR, Li CY, Liou MJ, Dung NX, Wu TS.* New diterpenoids and the bioactivity of *Erythrophleum fordii*. Bioorg Med Chem 2008; 16: 9867–9870
11 *Miyagawa T, Ohtsuki T, Koyano T, Kowithayakorn T, Ishibashi M.* Cassaine diterpenoid dimers isolated from *Erythrophleum succirubrum* with TRAIL-resistance overcoming activity. Tetrahedron Lett 2009; 50: 4658–4662
12 *Hara S, Okabe H, Mhashi K.* Gas-liquid chromatographic separation of aldose enantiomers as trimethylsilyl ethers of methyl 2-(polyhydroxyalkyl)-thiazolidine-4(*R*)-carboxylates. Chem Pharm Bull 1987; 35: 501–506
13 *Liu R, Ma SG, Yu SS, Pei YH, Zhang S, Chen XG, Zhang JJ.* Cytotoxic oleanane triterpene saponins from *Albizia chinensis*. J Nat Prod 2009; 72: 632–639
14 *Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR.* Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res 1988; 48: 589–601
15 *Braca A, Abdel-Razik AF, Mendez J, Tommasi ND.* Triterpenoid saponins from *Campsiandra guayanensis*. J Nat Prod 2006; 69: 240–246
16 *Yaguchi Y, Sakurai N, Nagai M, Inoue T.* Constituents of *Myrica rubra*. III. Structures of two glycosides of myricanol. Chem Pharm Bull 1988; 36: 1419–1424
17 *Tchivounda HP, Koudogbo B, Besace Y, Casadevall E.* Triterpene saponins from *Cylicodiscus gabunensis*. Phytochemistry 1991; 30: 2711–2716
18 *Loder JW, Nearn RH.* Tumour inhibitory plants. New alkaloids from the bark of *Erythrophleum chlorostachys* (Leguminosae). Tetrahedron Lett 1975; 29: 2497–2498