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Erythrocarpines A–E, new cytotoxic limonoids from *Chisocheton erythrocarpus*

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Abstract—Five new cytotoxic limonoids, erythrocarpines A–E (**1**–**5**), were isolated from the bark of *Chisocheton erythrocarpus* Hiern. Chemical structures, stereochemistry, and conformation were fully elucidated and characterized by 2D NMR, MS, and computational methods.

The plants from family Meliaceae are known to produce various types of cytotoxic tetranortriterpenoids known as limonoids.[1,2] Two widely known limonoids are azadirachtin from *Azadirachta indica* and toosendanin from *Melia toosendan*.[3,4] Both are used as insecticides and have been commercialized in US and China.

In continuation of our research on this family, we have embarked a study on the dichloromethane extract of the bark of *Chisocheton erythrocarpus* Hiern (Meliaceae) and five new limonoids, erythrocarpines A–E (**1**–**5**), were isolated. This paper describes the structural elucidation of erythrocarpines A–C (**1**–**3**) belonging to mexicanolide-type limonoid and erythrocarpines D (**4**) and E (**5**) having A, B, D-*seco* heptacyclic skeletons, which showed cytotoxicity against P-388 murine leukemia cells.

The bark was defatted with hexane and then extracted with dichloromethane. The crude dichloromethane extract showed cytotoxic activity against P388 murine leukemia cells at IC_{50} 18 μg/ml and was subjected to silica gel column chromatography, PTLC, and HPLC to yield five new limonoids.

Erythrocarpine A (**1**), $[\alpha]_D$ +95° (*c* 1.9, $CHCl_3$), was isolated as white amorphous powder. The HRFABMS showed a $[M+H]^+$ peak at *m/z* 573.2491, corresponding to the molecular formula of $C_{34}H_{36}O_8$. IR absorption implied the presence of esteric ketone (1746 cm^{-1}) groups. The ^1H NMR spectrum showed the presence of the characteristic H-17 singlet at δ_H 5.08, four methyl singlets at δ_H 0.95 (Me-18), 1.16 (Me-19), 0.82 (Me-28), and 0.80 (Me-29), and a methoxy singlet (δ_H 3.64). The presence of a β-furyl ring was represented by H-21 at δ_H 7.35 (d, $J = 0.8$ Hz), H-22 at δ_H 6.40 (dd, $J = 0.8$, 1.7 Hz), and H-23 at δ_H 7.35 (d, $J = 1.7$). Two double bond proton signals, H-15 and H-30, were detected at δ_H 5.93 (s) and δ_H 6.22 (dd, $J = 2.9$, 6.1 Hz), respectively. A benzoyl group was found by the detection of five aromatic protons in the region δ_H 7.40–8.10 and a carbon at δ_C 165.5 (C-1′). In addition, the ^{13}C NMR spectrum indicated the presence of two carbonyls of a cyclohexanone at δ_C 214.5 (C-1) and a lactone of C-16 at δ_C 164.6.

Figure 1 shows selected 2D NMR correlations for erythrocarpine A (**1**). HMBC correlations of H-17 to C-20, C-21, and C-22 indicated the presence of a β-furyl ring at C-17. HMBC correlations of the methoxy peak

Keywords: Meliaceae; Limonoids; P388 murine leukemia cell; Structural elucidation.
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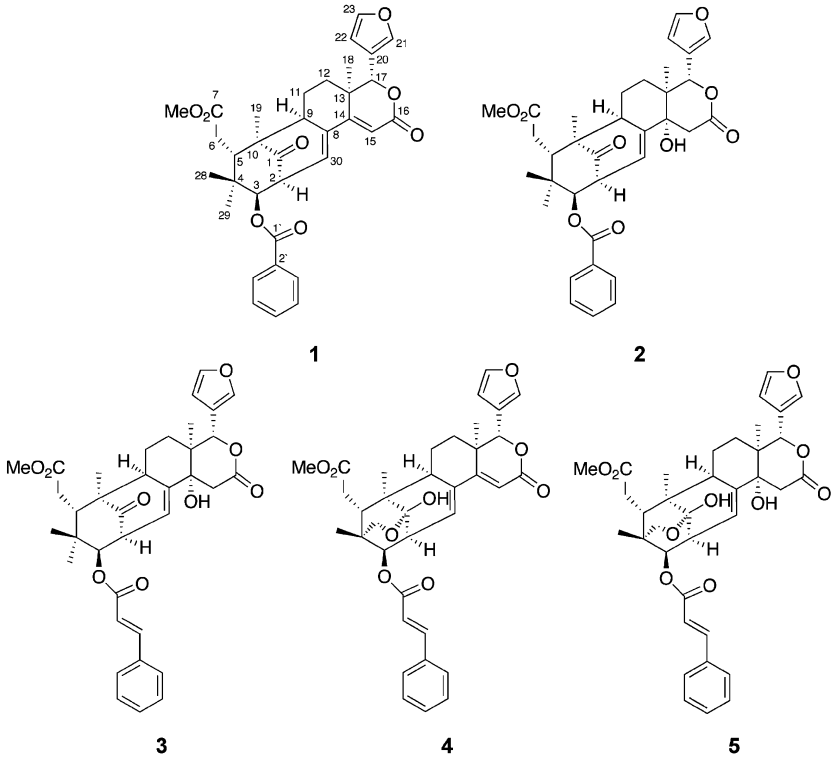

at δ_H 3.64 and H_2-6 to C-7 suggested that the methoxy group was attached to C-7. The position of Δ^{8-30} double bond was confirmed by the HMBC correlations of H-2 to C-1 (δ_C 214.5), C-8 (δ_C 136.2), and C-30 (δ_C 128.8), and H-30 to C-9 (δ_C 54.0) and C-14 (δ_C 160.3). In the δ-lactone ring (ring-D), the vinylic proton of H-15 showed the HMBC correlations to both bridgehead carbons of C-13 (δ_C 37.4) and C-14 (δ_C 160.3). This δ-lactone substructure was conjugated to the Δ^{8-30} double bond to form a conjugated diene lactone system, which was responsible for the down-field proton signals of H-15 (δ_H 5.93) and H-30 (δ_H 6.22).[5] The presence of a benzoyl group at C-3 was confirmed based on the HMBC correlation of H-3 (δ_H 5.11) to C-1′ (δ_C 165.5). Two methyls of C-18 and C-19 were attached to C-13 and C-10, respectively, by HMBC correlations of H_3-18 to C-12, C-13, and C-17, and of H_3-19 to C-5, C-9, and C-10. Thus, the gross structure of erythrocarpine A (**1**) was suggested to possess mexicanolide-type skeleton with a β-furyl ring, a conjugated δ-lactone, and a benzoate as shown in Figure 1.

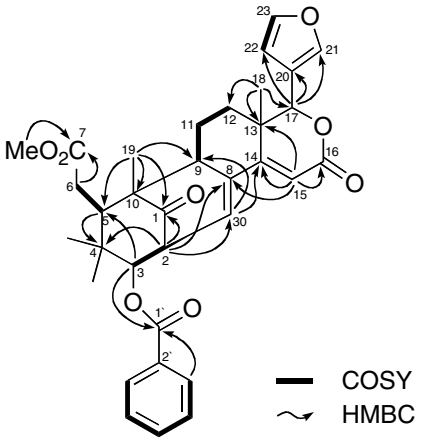

Figure 1. Selected 2D NMR correlations of erythrocarpine A (**1**).

The relative stereostructure of **1** was elucidated by NOESY cross-peaks as depicted in the computer-generated 3D drawing, which was consistent with the calculated energy minima as shown in Figure 2. Cross-peak between H-5 and H-11β suggested that the former was at β orientation and ring C was in conformation of skew boat form. In addition, the NOESY spectrum also showed a correlation between H-9 and H-11α, thus suggesting an α orientation for H-9. The relative configurations at C-2 and C-3 were deduced by the NOESY correlation between H-2 and H-3 together with their ^3J coupling constant (J = 10.5 Hz). Thus, the relative stereostructure of erythrocarpine A (**1**) was depicted as in Figure 2.

The conformational space for **1** was searched using MMFF force field[6] implemented in the Macromodel program,[7] and the results of the NMR study described above were considered in terms of these calculations. Each of the lowest energy conformers belonging to four separate clusters is represented as **A** (471.9 kJ/mol), **B** (470.9 kJ/mol), **C** (474.8 kJ/mol), and **D** (481.6 kJ/mol). The ring A of conformers **A** and **C** possessed a twist boat form in the bicyclo[3.3.1]nonane part, while conformers **B** and **D** adopted a twist chair form (Fig. 3). The populations calculated for these four clusters implied that **A** and **B** were abundant. This result was consistent with the presence of the NOESY correlation between H-17 and H-11β, whereas there was no NOESY correlation between Me-28 and H_2-11 in conformer **A**.

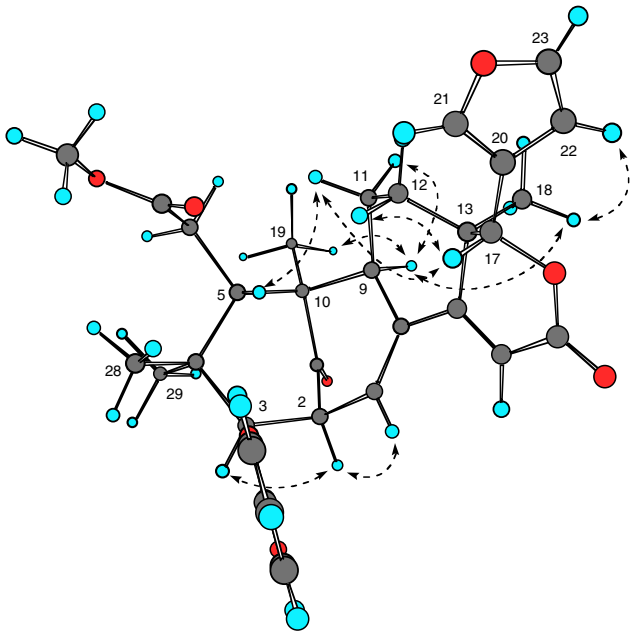

Figure 2. Selected NOESY correlations of erythrocarpine A (**1**).

These NMR data indicated that erythrocarpine A (**1**) took a most probable conformer **A** in solution.[8]

Erythrocarpine B (**2**), $[\alpha]_D$ −139° (*c* 1.9, $CHCl_3$), revealed a $[M+H]^+$ peak at *m/z* 591.2600 in the HRFABMS, corresponding to the molecular formula of $C_{34}H_{38}O_9$. The ^1H and ^{13}C NMR spectra were similar to those of **1**, except for the absence of Δ^{14-15} double bond. Without the conjugative effect as in **1**, H-30 was significantly shifted upfield to δ_H 5.69. The C-14 bridgehead carbon bearing a hydroxyl group was observed at δ_C 73.3, which was correlated with H-17, H-9, and H-30 in the HMBC spectrum. Therefore, **2** was actually a derivative of **1** through hydroxylation at Δ^{14-15} double bond. The relative stereochemistry and α orientation of the hydroxyl at C-14 were elucidated by NOESY correlations in Figure 4.

Erythrocarpine C (**3**), $[\alpha]_D$ −251° (*c* 0.9, $CHCl_3$), has a molecular formula of $C_{36}H_{40}O_9$, determined from the $[M+H]^+$ peak at *m/z* 617.2759 in the HRFABMS. The ^1H and ^{13}C NMR spectra were almost the same as those of **2**, which suggested that **2** and **3** possessed the same basic skeleton and stereochemistry. However, a cinnamoyl group was detected instead of a benzoyl group at C-3. This was confirmed by the presence of five aromatic protons in the region δ_H 7.20–7.60, and the characteristic doublet signals of H-2′ and H-3′ at δ_H 6.39 (d, J = 16.0 Hz) and δ_H 7.68 (d, J = 16.0 Hz), respectively.[9]

Erythrocarpine D (**4**), $[\alpha]_D$ −98° (*c* 1.9, $CHCl_3$), showed a $[M+H]^+$ peak at *m/z* 615.2594 in the HRFABMS, suggesting a molecular formula of $C_{36}H_{38}O_9$. The ^1H and ^{13}C NMR spectra showed the presence of functionalities such as a β-furyl ring, a methoxy group, a conjugated δ-lactone ring, and a cinnamoyl group. The Me-29 was suggested to be oxidized and cyclized with C-1 to form

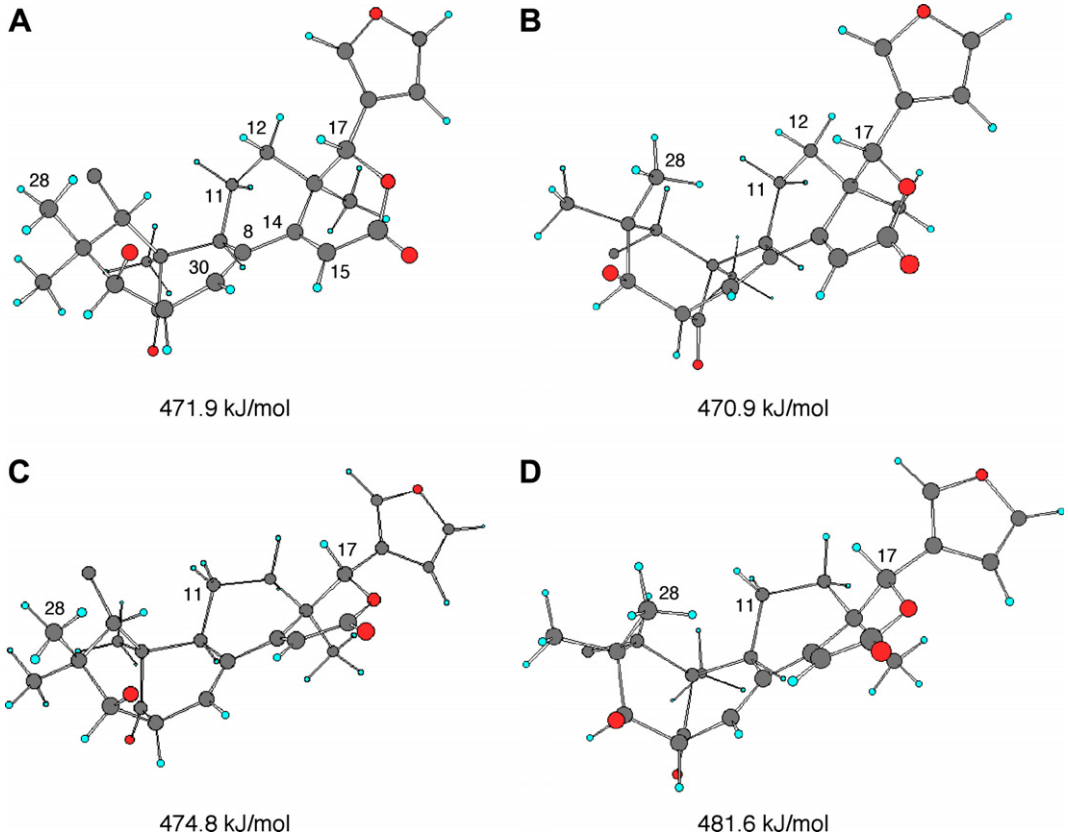

Figure 3. Four representative stable conformers (A–D) of erythrocarpine A (**1**) analyzed by Monte Carlo simulation followed by minimization and clustering analysis. Benzoate and methoxy carbonyl functions at C-3 and C-6, respectively, were omitted for clarity.

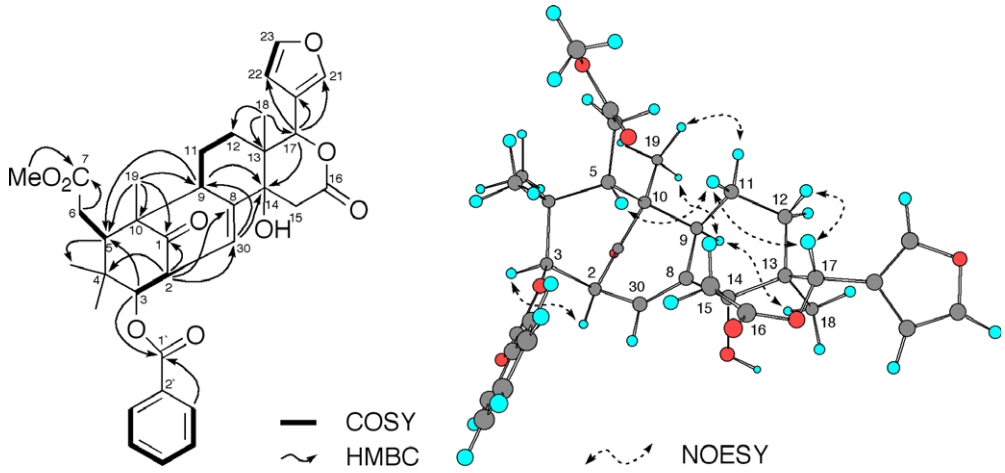

Figure 4. Selected 2D NMR correlations of erythrocarpine B (**2**).

Table 1. Cytotoxicity of erythrocarpines A–E (**1**–**5**) against P388 murine leukemia cells

Compound	IC_{50} (μg/ml)
Erythrocarpine A (**1**)	2.0
Erythrocarpine B (**2**)	6.0
Erythrocarpine C (**3**)	9.9
Erythrocarpine D (**4**)	10.0
Erythrocarpine E (**5**)	16.0
MMC (mitomycin C)	0.15

an oxygen-bridge in the ring A.[10] It was opposed to the typical 4,29,1-carbon-bridge sequence found in the phragmalin skeleton which was biogenetically and structurally closely related to mexicanolide skeleton. The connectivity was established through HMBC correlations of H_2-29 to C-1 (δ_C 96.4), C-3 (δ_C 76.1), C-4 (δ_C 36.3), and C-5 (δ_C 33.7).

Erythrocarpine E (**5**), $[\alpha]_D$ −128° (*c* 0.8, $CHCl_3$), has a molecular formula of $C_{36}H_{40}O_{10}$, established from a

Table 2. ^1H NMR data [δ_H (*J*, Hz)] of erythrocarpines A–E (**1**–**5**) in $CDCl_3$

	1	**2**	**3**	**4**	**5**
2	3.78 dd (6.1, 10.5)	3.53 m	3.53 m	3.18 dd (2.7, 10.0)	2.95 dd (7.1, 10.0)
3	5.11 d (10.5)	5.14 d (9.4)	5.03 d (9.5)	5.00 d (10.0)	4.91 d (10.2)
5	3.39 dd (1.7, 9.5)	3.50 m	3.50 m	2.80 d (10.5)	2.92 m
6	2.26 dd (1.7, 16.8)	2.41 br d (8.7)	2.40 m	2.32 m	2.31 m
6	2.31 dd (9.8, 16.9)	2.43 m	2.45 m	2.44 m	2.41 m
9	2.19 br d (12.4)	2.74 m	2.73 dd (5.6, 12.5)	2.37 m	2.34 dd (3.2, 10.2)
11a	1.70 m	2.07 m	2.11 m	1.72 m	1.54 m
11b	1.49 m	1.55 m	1.67 m	1.34 m	1.68 m
12a	1.18 m	1.33 br d (13.8)	1.35 br d (14.1)	1.39 m	1.17 m
12b	1.86 m	1.96 m	1.98 td (4.7, 14.1)	1.81 m	1.89 m
15	5.93 s	2.76 d (18.3)	2.85 d (17.9)	6.13 s	2.76 d (18.3)
15		2.87 d (18.3)	2.96 d (17.9)		2.86 d (17.8)
17	5.08 s	5.69 s	5.76 s	5.06 s	5.46 s
18	0.95 s	1.03 s	1.04 s	1.07 s	0.88 s
19	1.16 s	1.19 s	1.18 s	1.13 s	1.02 s
21	7.35 d (0.8)	7.91 br s	7.84 br s	7.45 s	7.64 s
22	6.40 dd (0.8, 1.7)	6.51 br s	6.49 br s	6.45 s	6.29 br s
23	7.35 d (1.7)	7.45 br s	7.43 br s	7.39 m	7.30 d (1.5)
28	0.82 s	0.89 s	0.95 s	0.68 s	0.62 s
29	0.80 s	0.86 s	0.82 s	3.49 dd (1.4, 9.5)	3.46 d (8.8)
29				3.97 d (9.8)	3.88 d (9.8)
30	6.22 dd (2.9, 6.1)	5.69 d (9.1)	5.73 dd (1.5, 6.8)	6.30 dd (2.7, 6.1)	5.53 dd (1.7, 7.1)
7-OMe	3.64 s	3.73 s	3.72 s	3.69 s	3.65 s
2′			6.39 d (16.0)	6.49 d (16.1)	6.25 d (15.5)
3′	8.01 d (8.3)	7.98 m	7.68 d (16.0)	7.80 d (16.1)	7.58 d (15.9)
4′	7.44 m	7.38 m			
5′	7.56 m	7.52 m	7.52 m	7.57 m	7.19 m
6′	7.44 m	7.38 m	7.29 m	7.41 m	7.18 m
7′	8.01 d (8.3)	7.98 m	7.28 m	7.41 m	7.19 m
8′			7.29 m	7.41 m	7.18 m
9′			7.52 m	7.57 m	7.19 m

$[M+H]^+$ peak at *m/z* 633.2703 in the HRFABMS. Comparison of NMR data with those of **1**–**4** established the substitution pattern of **5** to have a similar tricyclodecane system as in **4**, allyl alcohol sequence as in **2** and **3**, and 3-cinnamoyl moiety as in **3** and **4**.

Erythrocarpines A–E (**1**–**5**) are A, B, D-*seco* heptacyclic limonoids having mexicanolide-type skeleton with either benzoyl or cinnamoyl group as side chains at C-3. All of them showed cytotoxicity against P388 murine leukemia cells as shown in Table 1. It was noted that erythrocarpines A (**1**) and B (**2**) with a benzoyl side chain exhibited more potent activities than erythrocarpines C (**3**)–E (**5**) with a cinnamoyl side chain (Table 1).

1. Experimental

1.1. General methods

Merck silica gel 60 (230–400 mesh) and GF_{254} were used for column chromatography separations, silica gel 60 F_{254} for TLC, silica gel 604 F_{254} for PTLC, and CAPCELL PAK C18 Type AQ 5 μm, 4.6 mm × 250 mm for HPLC separation. NMR spectra were recorded on JEOL JNM-FX100 (400 MHz) using $CDCl_3$. The IR spectra were measured using Perkin-Elmer 1600 Double Beam.

1.2. Material

The barks of *C. erythrocarpus* Hiern were collected at Hutan Simpan Terenas, Malaysia. The botanical identification was made by Mr. Teo Leong Eng, Faculty of Science, University of Malaya. Voucher specimens are deposited in the Herbarium of Chemistry Department, University of Malaya.

1.3. Extraction and isolation

Dried ground barks (1.3 kg) were extracted successively with hexane, dichloromethane, and methanol. The dichloromethane extract (4.0 g) was repeatedly subjected to silica gel column chromatography (CC) using solvent mixture of hexane: ethyl acetate (85:15) to yield **1** (26.1 mg). The fraction eluted with hexane/ethyl acetate (7:3) was furthermore purified by HPLC using MeOH/H_2O (4:1) to yield **2** (3.6 mg) and **3** (7.2 mg), and by repeated CC and PTLC using hexane: ethyl acetate solvent system to afford **4** (14.0 mg) and **5** (4.7 mg).

Erythrocarpine A (**1**). Amorphous powder, $[\alpha]_D$ +95° (*c* 1.9, $CHCl_3$); HRFABMS *m/z* 573.2491 $[M+H]^+$ (calcd for $C_{34}H_{37}O_8$, 573.2488); UV (MeOH) λ_{max}: 300 nm (ε 4000); CD (MeOH) $[\theta]_{220}$ −2000, $[\theta]_{238}$ +4000, and $[\theta]_{300}$ +10300; IR ($CHCl_3$) cm^{-1}: 2951, 1746, 1598, and 1264; ^1H and ^{13}C NMR: Tables 2 and 3.

Erythrocarpine B (**2**). Amorphous powder, $[\alpha]_D$ −139° (*c* 1.9, $CHCl_3$); HRFABMS *m/z* 591.2600 (calcd for $C_{34}H_{39}O_9$, 591.2594); UV (MeOH) λ_{max}: 300 nm; IR ($CHCl_3$) cm^{-1}: 3423, 1714, 1620, and 1231; ^1H and ^{13}C NMR: Tables 2 and 3.

Table 3. ^{13}C NMR data [δ_C] of erythrocarpines A–E (**1**–**5**) in $CDCl_3$

C	1	2	3	4	5
1	214.5	216.7	216.7	96.4	97.3
2	49.0	48.9	48.7	45.4	44.8
3	78.6	77.0	77.0	76.1	74.9
4	39.0	38.8	39.1	36.3	36.2
5	40.3	41.3	41.6	33.7	34.9
6	32.8	32.9	33.0	31.9	31.7
7	174.3	174.1	174.1	173.7	174.0
8	136.2	141.5	140.8	135.3	140.5
9	54.0	52.3	52.3	44.4	43.6
10	51.9	50.0	50.1	42.0	41.4
11	21.7	20.2	20.0	19.8	19.0
12	32.8	28.6	28.4	32.4	28.2
13	37.4	41.1	41.2	37.4	41.2
14	160.3	73.3	73.2	161.9	78.9
15	112.4	38.8	38.6	110.8	39.4
16	164.6	167.6	168.0	165.2	168.8
17	79.5	77.3	77.2	79.8	76.9
18	22.4	15.6	15.8	21.5	14.8
19	15.6	15.9	15.9	14.4	14.7
20	120.2	120.2	120.3	120.3	120.3
21	141.4	142.3	142.1	141.3	146.2
22	110.2	110.0	110.1	110.2	109.8
23	143.1	142.9	142.8	143.0	142.6
28	21.0	20.3	20.3	14.7	15.5
29	22.1	22.5	22.4	68.1	67.9
30	128.8	124.4	124.9	127.0	122.1
7-OMe	52.0	53.0	52.8	52.1	52.1
1′	165.5	165.8	166.3	166.5	166.4
2′	129.6	128.8	116.9	116.7	117.0
3′	128.9	129.7	146.5	146.7	146.2
4′	128.9	128.8	130.2	134.0	134.4
5′	133.9	133.5	128.7	128.4	128.3
6′	128.9	128.8	128.6	128.9	128.5
7′	129.6	129.7	134.4	130.7	129.9
8′			128.6	128.9	128.5
9′			128.7	128.4	128.3

Erythrocarpine C (**3**). Amorphous powder, $[\alpha]_D$ −251° (*c* 0.9, $CHCl_3$); HRFABMS *m/z* 617.2759 (calcd for $C_{36}H_{41}O_9$, 617.2751); UV (MeOH) λ_{max}: 300 nm; IR ($CHCl_3$) cm^{-1}: 3421, 1712, 1626, and 1231; ^1H and ^{13}C NMR: Tables 2 and 3.

Erythrocarpine D (**4**). Amorphous powder, $[\alpha]_D$ −98° (*c* 1.9, $CHCl_3$); HRFABMS *m/z* 615.2594 $[M+H]^+$ (calcd for $C_{36}H_{39}O_9$, 615.2594); UV (MeOH) λ_{max}: 296 nm; IR ($CHCl_3$) cm^{-1}: 3009, 1706, 1611, and 1241; ^1H and ^{13}C NMR: Tables 2 and 3.

Erythrocarpine E (**5**). Amorphous powder, $[\alpha]_D$ −128° (*c* 0.8, $CHCl_3$); HRFABMS *m/z* 633.2703 (calcd for $C_{36}H_{41}O_{10}$, 633.2700); UV (MeOH) λ_{max}: 297 nm; IR ($CHCl_3$) cm^{-1}: 3413, 2928, 1700, and 1201; ^1H and ^{13}C NMR: Tables 2 and 3.

1.4. Cytotoxic assays

P-388 murine leukemia cells were maintained in RPMI-1640 medium supplemented with 5% fetal calf serum and kanamycin (100 μg/ml). The cells (3×10^3 cells/well) were cultured in Corning disposable 96-well plates containing 100 μl of growth medium per well and were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Various drug concentrations (10 μl) were added to the

cultures at day one after the transplantation. At day three, 20 μl MTT solution (5 mg/ml) per well was added to each cultured medium. After a further 4 hours of incubation, 100 ml of 10% SDS–0.01 N HCl solution was added to each well and the formazan crystals in each well were dissolved by stirring with a pipette. The optical density measurements were made using a micropipette reader (Tohso MPR-A4i) with a two wavelength system (550 and 700 nm). In all experiments, three replicate wells were used to determine each point.

1.5. Computational methods

Conformational searching was carried out using Monte Carlo simulation in Macromodel program for erythrocarpine A (**1**).[7] The closure bonds for the skeleton of **1** were chosen at C-5–C-10, C-2–C-30, C-9–C-11, and C-13–C-17 with the closure limit from 1 to 4 Å. Three thousand Monte Carlo steps were performed, yielding 144 unique conformations in the energy region of 0–10 kcal/mol, which could be classified into four clusters. Each conformer was finally minimized by molecular mechanics calculation of MMFF force field.[6] The lowest energy conformation of each cluster is depicted in Figure 1.

Acknowledgments

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References and notes

1. Champagne, D. E.; Koul, O.; Isman, M. B.; Scudder, G. G. E.; Towers, G. H. N. *Phytochemistry* **1992**, *31*, 377–394.
2. Da Silva, M. F. D. G. F.; Gottlieb, O. R.; Dreyer, D. L. *Biochem. Syst. Ecol.* **1984**, *12*, 299–310.
3. Nathan, S. S.; Kalaivani, K.; Chung, P. G. *Pest. Biochem. Physiol.* **2005**, *81*, 46–57.
4. Tada, K.; Takido, M.; Kitanaka, S. *Phytochemistry* **1999**, *51*, 787–791.
5. Abdelgaleil, S. A. M.; Iwagawa, T.; Doe, M.; Nakatani, M. *Fitoterapia* **2004**, *75*, 566–572.
6. Halgren, T. *J. Am. Chem. Soc.* **1990**, *112*, 4710–4723.
7. Monte Carlo simulation and molecular mechanics calculation were conducted by Macromodel program: Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440–467.
8. Positive Cotton curve at 238 nm and negative one at 220 nm, which may be due to positive helicity in the diene system of C-15, C-14, C-8, and C-30 in conformer **A**, was observed in the CD spectrum of erythrocarpine A (**1**).
9. Zheng, L.; Gu, Z. M.; Fang, X. P.; Fanwick, P. E.; Chang, C. J.; Smith, D. L.; Mclaughlin, J. L. *Tetrahedron* **1995**, *51*, 2477–2488.
10. Wu, J.; Zhang, S.; Xiao, Q.; Li, Q.; Huang, J.; Long, L.; Huang, L. *Tetrahedron lett.* **2004**, *45*, 591–593.