Studies on Anti-cancerous and Anti-malarial Substances from Simaroubaceae Plants

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Summary

Cancer is a general term subjected to a series of malignant tumor diseases which may affect many different parts of the human body. These cancer diseases are characterized by a rapid and uncontrolled formation of abnormal cells in the body. Cancer chemotherapeutic agents can often provide the prolongation of life and occasionally cures. To date many kinds of compounds have been obtained from plants kingdom as anti-neoplastic and anti-cancerous agents. However, there is no special type of compounds for cancer therapy. In our laboratory, anti-tumor and cytotoxic screenings on higher plants collected in Japan, China, Korea, Southeast Asia and South America have been done by using Sarcoma 180 ascites in mice, P388 lymphocytic leukemia in mice, Chinese hamster lung V-79 cells, P388 cells and nasopharynx carcinoma (KB) cells.

The family, Simaroubaceae consists of about 20 genera and 120 species, mainly shrubs and trees, distributed in tropical and subtropical country. Simaroubaceae is classified as RUTALES, together with Rutaceae, Burseraceae, Meliaceae, Malpighiaceae and Polygalaceae. The members differ from the Rutaceae in not containing oil glands. Bitter principles are a characteristic of the family, Simaroubaceae. The genera include *Quassia* (*Simarouba*) (40 spp.), *Picrasma* (*Aeschrion*) (6 spp.), *Brucea* (10 spp.), *Soulamea* (10 spp.), *Ailanthus* (10 spp.) and *Perriera* (1 spp.) etc.. Surinam quassia derived from *Quassia amara* growing in Guianas, north Brazil and Venezuela is used in traditional medicines for stomachic, anti-amoebic, anti-malarial and anti-anaemic properties Also, various parts of a number of plants of the family Simaroubaceae have been used in traditional medicine for the treatment of a variety of diseases including cancer, amoebic, dysentery and malaria Then, the research has established that it is the quassinoid content of these plants that is responsible for above activities.

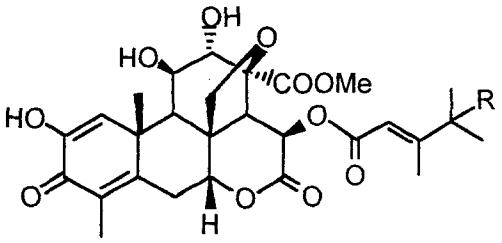
In this meeting, I will present on anti-tumor and anti-malarial activities and their active principles of Simaroubaceae plants, *Eurycoma longifolia, Ailanthus vilmoriniana, Simaba cedron* and *Brucea mollis* which have been studied in our laboratory.

Quassinoids from *Brucea mollis* Wall.

Keywords——*Brucea mollis*; cytotoxicity; quassinoid; Simaroubaceae

In our investigation of antitumor substances from higher plants,[1] we found that a methanolic extract of *Brucea mollis* Wall. (Simaroubaceae) which is used as an antimalarial agent and parasiticide in the folk medicine in south China[2] had a potent cytotoxic activity. The present paper deals with the isolation and structural determination of the active principles of the extract.

A methanolic extract (358 g) prepared from the bark (4 kg) of *B. mollis*, which was collected in the Xi Shuang Ban Na area of Yun Nan Province and identified by Dr. Guo-da Tao, Kungming Institute of Botany, Academia Sinica, was diluted with water and extracted first with methylene chloride, and then with *n*-butanol. The methylene chloride fraction (92.6 g), having most of the cytotoxic activity of the extract, was subjected to chromatographic separations by monitoring the cytotoxic effect on lymphocytic leukemia P388 cells to isolate the active principles. The final purification was made by ODS HPLC to give four known quassinoids, dehydrobruceantinol (**1**, 78 mg), bruceantinol (**2**, 58 mg), dehydrobruceantin (**3**, 11 mg) and bruceantin (**4**, 2 mg). Those structures were confirmed by comparing their physical and spectral data with those of the literature.[3-6]



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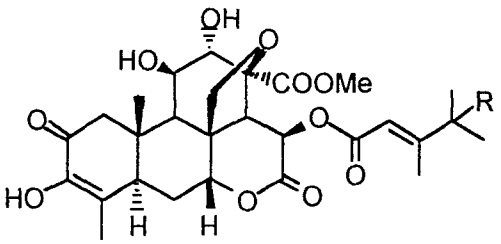
3: R=H



2: R=OAc, IC_{50}=0.013 μg/ml
4: R=H

References and Notes

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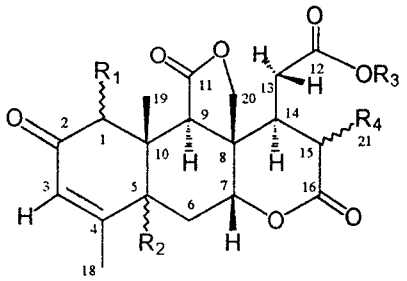
QUASSINOIDS FROM *AILANTHUS VILMORINIANA*

Key Word Index —— *Ailanthus vilmoriniana*; Simaroubaceae; quassinoid; vilmorinine; spectroscopic analysis.

Abstract —— The new quassinoids, named vilmorinines B (**1**) - F (**5**), have been isolated from the cortex of *Ailanthus vilmoriniana* (Simaroubaceae). Their structures were established by various spectroscopic evidences.

INTRODUCTION

During our survey of new antitumour substances from higher plants [1], especially Simaroubaceae [2 - 6], the crude extract of *Ailanthus vilmoriniana* (Simaroubaceae) showed cytotoxic activity against P388 leukemia cells. In previous studies [7], a new quassinoid named vilmorinine A was obtained from this plant. The further investigation led us to isolate five novel quassinoids, vilmorinines B (**1**) - F (**5**). In this paper, their structural elucidation is reported.



vilmorinine B (**1**): R_1=β-OH, R_2=α-H, R_3=Me, R_4=β-Me
vilmorinine C (**2**): R_1=β-OH, R_2=α-H, R_3=H, R_4=β-Me
vilmorinine D (**3**): R_1=α-OH, R_2=α-H, R_3=H, R_4=β-Me
vilmorinine E (**4**): R_1=β-OH, R_2=β-H, R_3=H, R_4=β-Me
vilmorinine F (**5**): R_1=β-OH, R_2=β-H, R_3=H, R_4=α-Me

Fig. 1 Quassinoids isolated from *Ailanthus vilmoriniana*

RESULTS AND DISCUSSION

The methanolic extract prepared from the cortex of A. vilmoriniana was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 soluble material was subjected to silica gel column chromatography (CH_2Cl_2 - MeOH) to give eight fractions. Further purification of each fraction

using silica gel open column (n-hex - EtOAc), MPLC and HPLC on ODS column (MeOH - H_2O) furnished five new quassinoids, vilmorinines B (**1**) - F (**5**).

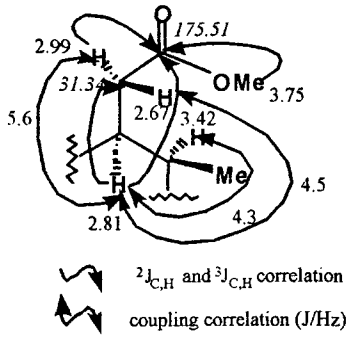


Fig. 2 Partial structure of vilmorinine B (**1**)

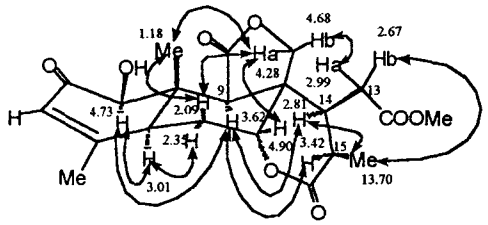


Fig. 3 NOE correlationof vilmorinine B(**1**)

Vilmorinine B (**1**) was colourless and showed partial structures of an α,β-unsaturated carbonyl group, two lactone and one ester carbonyl groups in IR, UV and ^{13}C-NMR. Further, the proton signals of 19-Me, 21-Me, 18-Me, OMe, 3-olefinic H and 20-H_2 were observed. From above data, and the ^{1}H-^{1}H and ^{1}H-^{13}C long range coupling correlations in Fig. 2, **1** was characterized as a C_{11}-C_{12} bond-cleaved quassinoid shown in Fig. 1. Also, stereochemistry of **1** was established as shown in Fig. 3 by the correlations of NOESY spectrum.

Vilmorinine C (**2**) was characterized as colourless needles. The IR, UV, MS and NMR spectral data of **2** were similar to those of **1**, but no OMe signal was observed in the NMR spectrum of **2** and the molecular ion peak was 14 mass unit less than that of **1**. These findings show that vilmorinine C (**2**) is characterized as shown in Fig. 1.

Vilmorinine D (**3**) was characterized as colourless needles and the molecular ion peak was same to that of **2**. The IR, UV, MS and NMR spectral data of **3** were similar to those of **2**, but the C_1 chemical shift of **3** was shifted more upfield to 8.6 ppm than that of **2**. Consequently, vilmorinine D (**3**) was determined to be an 1-epimer of **2** as shown in Fig. 1.

Vilmorinine E (**4**) was characterized as colourless needles and the molecular ion peak was same to that of **2**. The IR, UV, MS and NMR spectral data of **4** were similar to those of **2**, but the NOE correlations between 19-Me and 5-H, 19-Me and 6β-H, 1-H and 6α-H, 1-H and 14-H, and 14-H and 6α-H as shown in Fig. 4 suggested that vilmorinine E (**4**) was a 5-epimer of **2**.

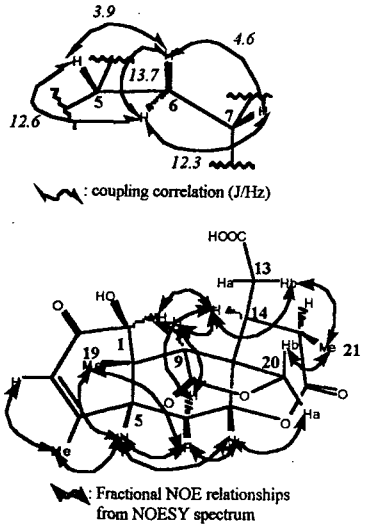


Fig. 4 ^{1}H-^{1}H Coupling and NOE correlation of vilmorinine E (4)

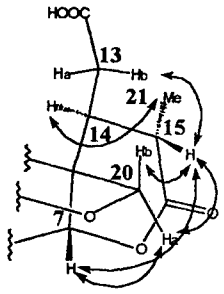


Fig. 5 Partial NOE correlation of vilmorinine F (5)

Vilmorinine F (**5**) was characterized as colourless needles and the molecular ion peak was same to that of **2**. The IR, UV, MS and NMR spectral data of **5** were similar to those of **4**, but the NOESY spectrum of **5** did not show the NOE correlations between 21-Me and 13β-H, and 21-H and 20-Hb which were observed in vilmorinines B (**1**) - E (**4**). Then, vilmorinine F (**5**) is confirmed to be a 15-epimer of **4** by the NOE correlations as shown in Fig. 5

Vilmorinines A (**1**) - F (**5**) isolated from *A. vilmoriniana* would be biosynthesized by lactonization between 7-OH and 12-COOH of a quassinoid as vilmorinine A [7] which is formed by an oxidative cleavage of the C_{11}-C_{12} bond of a quassinoid as chapparine [8].

EXPERIMENTAL

General. Mps: uncorr; ^{1}H and ^{13}C NMR: pyridine-d_5 with TMS as int. standard, Bruker AM400 or AM500. ^{13}C Multiplicities were determined by the DEPT pulse sequence. 2D-NMR (NOESY, HMQC, HMBC). EI-MS (70 eV) and FAB-MS (8 kV, glycerol), VG AutoSpec E or Finnigan MAT TSQ-700; IR (KBr or $CHCl_3$), Perkin-Elmer 1710 or JASCO A-302; UV (MeOH), Hitachi 557. Prep. HPLC: 10 μm ODS column; MPLC: 20 μm ODS column; Kieselgel 60; Anal. TLC: silica gel $60F_{254}$ and RP-18 F_{254} (0.25 mm) precoated plates. Spot detection: UV light at 254 nm and or spraying with 10% H_2SO_4.

Plant material. The cortices of *Ailanthus vilmoriniana* were collected at Emeishan, Sichuan

Province, People's Republic of China, in 1994. The botanical identification was made by Dr. Zhi-Sheng Qiao, Department of Pharmacognosy, College of Pharmacy, Second Military Medical University, Shanghai, China. A voucher specimen has been deposited in the herbarium of Tokyo University of Pharmacy & Life Science.

Table 1 ^{13}C and ^{1}H NMR chemical shifts(ppm) for vilmorinines B (1) - F (5)

	vilmorinine B (1)		vilmorinine C (2)		vilmorinine D (3)		vilmorinine E (4)		vilmorinine F (5)	
No.	δ_C	δ_H mult.(*J* /Hz)	δ_C	δ_H mult.(*J* /Hz)	δ_C	δ_H mult.(*J*/Hz)	δ_C	δ_H mult.(*J* /Hz)	δ_C	δ_H mult.(*J* /Hz)
1	84.65d	4.73s	84.58d	4.78s	76.00d	4.57s	75.22d	4.98s	74.82d	5.03s
2	197.49s		197.50s		196.92s		198.09s		198.28s	
3	126.88d	6.15brs	126.82d	6.13brs	125.55d	6.10brs	124.88d	6.00s	124.82d	6.03s
4	160.30s		160.49s		159.93s		160.87s		161.03s	
5	40.87d	3.01brd(12.5)	40.96d	3.08brd(12.6)	34.62d	3.35brd(13.7)	47.19d	2.63dd(12.6,3.9)	47.33d	2.61dd(12.6,4.2)
6	25.97t	2.35(α)ddd(15.6,2.4,2.4)	25.99t	2.36(α)brd(15.0)	26.12t	2.40(α)ddd(15.0,2.7,2.7)	30.82t	2.04(α)ddd(13.7,12.6,12.3)	31.51t	2.10(α)ddd(13.5,12.7,12.7)
		2.09(β)ddd(15.6,12.5,2.4)		2.10(β)ddd(15.0,12.6,2.4)		2.10(β)ddd(15.0,13.7,2.9)		2.33(β)ddd(13.7,4.6,3.9)		2.34(β)ddd(13.7,4.4,4.2)
7	75.64d	4.90brs	75.75d	4.97brs	76.38d	4.98brs	79.51d	4.80dd(12.3,4.6)	79.09d	4.78dd(12.2,4.4)
8	45.73s		45.80s		44.92s		46.24s		30.00s	
9	54.25d	3.62s	54.26d	3.82s	48.30d	4.23s	45.66d	3.88brs	46.22d	4.01s
10	44.99s		44.99s		42.34s		42.95s		42.82s	
11	172.72s		176.01s		176.69s		174.69s		175.15s	
12	175.51s		174.83s		174.94s		174.37s		170.00s	
13	31.34t	2.99(a)dd(17.1,4.5)	31.98t	3.02(a)dd(17.2,4.7)	32.16t	3.77(a)dd(16.9,4.7)	31.44t	3.84(a)m	34.51t	3.62(a)m
		2.67(b)dd(17.1,5.6)		2.78(b)dd(17.1,5.0)		3.03(b)dd(16.9,4.6)		2.96(b)dd(16.4,12.4)		2.76(b)dd(12.5,8.8)
14	40.57d	2.81ddd(5.6,4.5,4.3)	40.56d	2.95m	41.61d	3.03m	32.17d	3.80m	35.05d	3.58m
15	36.90d	3.42dq(6.5,4.3)	31.34d	3.47m	36.94d	3.42dqd(6.8,3.9)	36.66d	3.71m	40.75d	2.72m
16	172.23s		172.49s		172.34s		172.35s		171.47s	
18	22.15q	1.77s	22.16q	1.77s	22.16q	1.74s	22.31q	1.84s	22.38q	1.84s
19	10.70q	1.18s	10.66q	1.18s	14.82q	1.17s	18.14q	1.68s	17.98q	1.67s
20	69.41t	4.28(a)d(10.6)	69.67t	4.32(a)d(10.5)	72.81t	4.37(a)d(10.4)	67.45t	4.53(a)d(9.0)	67.27t	4.63(a)d(9.1)
		4.68(b)d(10.6)		4.83(b)d(10.5)		4.90(b)d(10.4)		4.41(b)d(9.0)		4.33(b)d(9.1)
21	13.70q	1.30d(6.5)	13.86q	1.42d(6.8)	13.74q	1.38d(6.8)	14.90q	1.41d(7.9)	15.17q	1.64d(6.8)
OMe	54.25q	3.75s								

Measurements were performed in pyridine-d_5 at 400MHz.
^{13}C Multiplicities were established by each DEPT pulse sequence.

Extract and isolation. The cortices of *A. vilmoriniana* (7.0 kg) were extracted with MeOH (30 *l*) three times. The MeOH extract (864 g) was partitioned between CH_2Cl_2 and H_2O. 95 g of CH_2Cl_2 soluble fraction (170 g) was subjected to si gel column chromatography using a CH_2Cl_2 - MeOH (1:0 - 0:1) gradient system to give 8 fractions.

The fourth fraction (78 g) was further applied to si gel column chromatography using a n-Hex - EtOAc (20:1 - 1:1) gradient system to furnish 7 fractions. The last fraction was applied to ODS MPLC and HPLC (MeOH - H_2O or MeCN - H_2O solvent system) to give vilmorinines C (**2**, 22.1 mg), D (**3**, 7.7 mg), E (**4**, 26.1 mg) and F (**5**, 2.3 mg).

The fifth fraction (23 g) was subjected to ODS MPLC using a MeOH - H_2O (9:11) solvent system to give 6 fractions. The first fraction was applied to si gel MPLC using a n-Hex - EtOAc (20:1 - 1:1) gradient system to give vilmorinine B (**1**, 45.8 mg).

Vilmorinine B (**1**). Colourless amorphous powder; mp 178 - 181°. $[\alpha]_D$ +40° (c 0.35,

MeOH); UV λ_{max} (MeOH) nm (log ε): 238 (3.6); IR ν^{CHCl_3} cm^{-1}: 3465, 1770, 1741, 1682, 1263, 1024; FAB-MS *m/z*: 407 ($[M+H]^+$, Calcd. for $C_{21}H_{27}O_8$: 407.1705, Found: 407.1709); ^1H and ^{13}C NMR (pyridine-d_5): Table 1.

Vilmorinine C (**2**). Colourless needles; mp 222 - 224°. $[\alpha]_D$ +14° (c 0.25, MeOH); UV λ_{max} (MeOH) nm (log ε): 238 (3.7); IR ν^{KBr} cm^{-1}: 3400, 1740, 1680, 1260, 1200; FAB-MS *m/z*: 393 ($[M+H]^+$, Calcd. for $C_{20}H_{25}O_8$: 393.1549, Found: 393.1520); ^1H and ^{13}C NMR (pyridine-d_5): Table 1.

Vilmorinine D (**3**). Colourless needles; mp 238 - 240°. $[\alpha]_D$ -51° (c 0.25, MeOH); UV λ_{max} (MeOH) nm (log ε): 240 (3.8); IR ν^{KBr} cm^{-1}: 3450, 1740, 1730, 1680, 1260, 1220; FAB-MS *m/z*: 393 ($[M+H]^+$, Calcd. for $C_{20}H_{25}O_8$: 393.1549, Found: 393.1535); ^1H and ^{13}C NMR (pyridine-d_5): Table 1.

Vilmorinine E (**4**). Colourless needles; mp 184 - 186°. $[\alpha]_D$ -34° (c 0.06, MeOH); UV λ_{max} (MeOH) nm (log ε): 237 (3.8); IR ν^{KBr} cm^{-1}: 3450, 1750, 1680, 1220; FAB-MS *m/z*: 393 ($[M+H]^+$, Calcd. for $C_{20}H_{25}O_8$: 393.1549, Found: 393.1554); ^1H and ^{13}C NMR (pyridine-d_5): Table 1.

Vilmorinine F (**5**). Colourless needles; mp 251 - 254°. $[\alpha]_D$ +67° (c 0.02, MeOH); UV λ_{max} (MeOH) nm (log ε): 238 (3.7); IR ν^{KBr} cm^{-1}: 3400, 1740, 1680, 1260, 1200; FAB-MS *m/z*: 393 ($[M+H]^+$, Calcd. for $C_{20}H_{25}O_8$: 393.1549, Found: 393.1547); ^1H and ^{13}C NMR (pyridine-d_5): Table 1.

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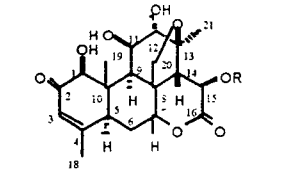
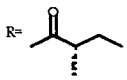
Cytotoxic Quassinoids from *Simaba cedron*

Four new quassinoids, cedronolactones A–D (**1**–**4**), together with nine known compounds, simalikalactone D (**5**), chaparrinone (**6**), chaparrin (**7**), glaucarubolone (**8**), glaucarubol (**9**), samaderine Z (**10**), guanepolide (**11**), ailanquassin A (**12**), and polyandrol (**13**), were isolated from the wood of *Simaba cedron*. The chemical structures of **1** - **4** were elucidated on the basis of their chemical and spectral properties. Cedronolactone A (**1**) was shown to exhibit a significant *in vitro* cytotoxicity (IC_{50} 0.0074 μg/mL) against P388 cells.

During a survey of new antitumor substances from higher plants,[1] especially those belonging to the Simaroubaceae,[2–8] we have found that the crude extract of *Simaba cedron* Planchon (Simaroubaceae) showed cytotoxic activity against P388 leukemia cells. Activity-guided chromatographic purification using P388 cells led to the isolation of four novel quassinoids, cedronolactones A–D (**1**-**4**) and nine known quassinoids, simalikalactone D (**5**),[9] chaparrinon (**6**),[10,11] chaparrine (**7**),[12] glaucarubolone (**8**),[10,13] glaucarubol (**9**),[14,15] samaderine Z (**10**),[16] guanepolide (**11**),[17] ailanquassin A (**12**),[18] and polyandrol (**13**).[19] In this paper, the structural elucidation of **1** - **4** and the cytotoxic activity of **1** - **13** are reported.

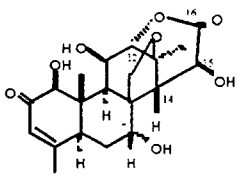


R= cedronolactone A (1)

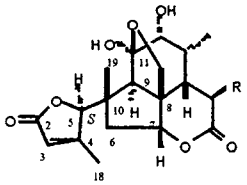


R= simalikalactone D (5)

R=H samaderine Z (10)

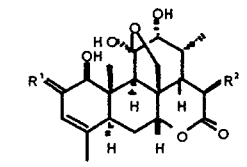


cedronolactone D (4)



R=H cedronolactone B (2)

R=OH cedronolactone C (3)

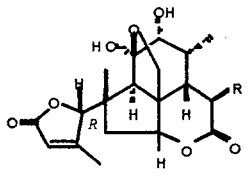


R^1 =O; R^2 =H chaparrinone (6)

R^1 =α-OH, β-H; R^2=H chaparrin (7)

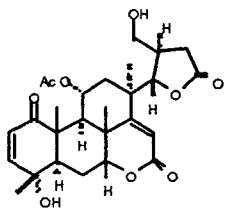
R^1 =O; R^2 =OH glaucarubolone (8)

R^1 =α-OH, β-H; R^2=OH glaucarubol (9)



R=H ailanquassin A (12)

R=OH polyandrol (13)



guanepolide (11)

Results and Discussion

The methanolic extract prepared from the wood of *S. cedron* was partitioned between $CHCl_3$ and H_2O, and then *n*-BuOH and H_2O. The $CHCl_3$-soluble material was subjected to silica gel column chromatography ($CHCl_3$–MeOH) to give eight fractions. Further purification of the fourth fraction using medium-pressure liquid chromatography (MPLC) (silica gel) and HPLC (ODS silica gel) furnished two new quassinoids, cedronolactones A (**1**) and B (**2**), and five known ones, simalikalactone D (**5**), chaparrinone (**6**), glaucarubolone (**8**), guanepolide (**11**), and ailanquassin (**12**). The *n*-BuOH soluble material was applied to Diaion HP-20 column chromatography (H_2O–MeOH). The fraction eluted with 20–60% MeOH was further chromatographed using MPLC and then HPLC to give the new quassinoids, cedronolactones C (**3**) and D (**4**), and known compounds, chaparrin (**7**), glaucarubolone (**8**), glaucarubol (**9**), samaderine Z (**10**), and polyandrol (**13**).

Cedronolactone A (**1**) was obtained as colorless needles, and its molecular formula was determined to be $C_{25}H_{34}O_9$ by HREIMS. Its IR, UV and ^{13}C-NMR spectra showed the presence of an α,β-unsaturated ketone, a δ-lactone, and an ester carbonyl group. The ^{1}H- and ^{13}C-NMR spectra of **1** were very similar to those of simalikalactone D (**5**)[9] except for the ester side chain moiety at the C-15. Analysis of the H-H COSY, HMBC and HMQC spectra revealed that compound **1** possesses a 3-methylbutanoyloxy group at C-15 position. From these data and NOESY spectra, the structure of cedronolactone A (**1**) was established as shown.

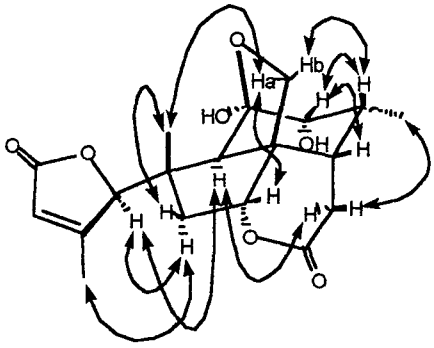


Figure 1. NOESY correlations of **2**.

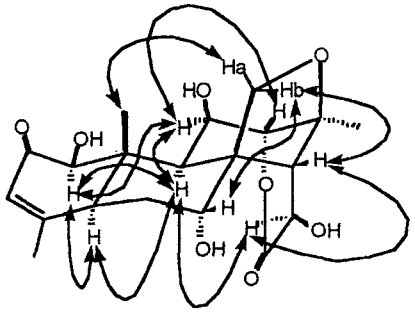


Figure 2. NOESY correlations of **4**.

Cedronolactone B (**2**) was characterized as colorless needles, whose molecular formula of $C_{19}H_{24}O_7$, as determined by HREIMS. The IR, UV and NMR spectral data showed the presence of

an α,β-unsaturated-γ-lactone and a δ-lactone, and were very similar to those of ailanquassin A (**12**).[18] However, the proton resonances of Me-18, H-6α and H-5 were observed at 0.44, 0.39 and 0.12 ppm more upfield, respectively, than analogous data for compound **12**. Furthermore, NOESY correlations were observed between H-5 and H-6α, H-5 and H-9, and H-6α and Me-18 as shown in Figure 1. These observations indicated that cedronolactone B (**2**) is the 5*S* epimer of **12**. This structure was confirmed by direct comparison with the authentic compound obtained by selective epimerization of **12** at the C-5 stereocenter.

Cedronolactone C (**3**) was characterized as colorless needles, and its molecular formula was determined by HREIMS as $C_{19}H_{24}O_8$. Although the IR, UV, MS, and NMR spectral data of **3** were similar to those of **2**, the presence of an additional hydroxyl group was suggested by its molecular formula and NMR spectra. The position of the hydroxyl group was determined by the shifts of H-15 (Δδ 2.12) and C-15 (Δδ 38.0) NMR resonances compared to those of **2**. Consequently, cedronolactone C (**3**) was deduced to be the 5*S* epimer of polyandrol (**13**).[19] The structure of **3** was confirmed by direct comparison with the authentic compound obtained by selective epimerization of **13** at C-5.

Cedronolactone D (**4**) was an characterized as amorphous solid, with its molecular formula determined as $C_{20}H_{26}O_8$ by HREIMS. Although its spectral data were similar to those of samaderine Z (**10**),[16] the C-7 and C-12 resonances of **4** were observed at δ 83.5 and δ 75.9, respectively, while those of **10** were observed at δ 72.8 and δ 87.0, respectively, in the ^{13}C-NMR spectrum. A long-range coupling was observed between H-12 and C-16 in the HMBC spectrum, which indicated that a lactone linkage exists between C-12 and C-16 in compound **4**. Furthermore, the NOESY correlation between H-9 and H-15α, as shown in Figure 2, suggested that the configuration of the hydroxyl group at the C-15 was in the β-configuration. From the above findings, structure **4** was deduced for cedronolactone D.

Compounds **5**–**13** were identified as simalikalactone D (**5**), chaparrinone (**6**), chaparrin (**7**), glaucarubolone (**8**), glaucarubol (**9**), samaderine Z (**10**), guanepolide (**11**), ailanquassin A (**12**), and polyandrol (**13**) respectively, by comparing their physical and spectral data with those reported in literature.[9–19]

The IC_{50} values (μg/mL) of compounds **1**–**13** against P388 lymphocytic leukemia cells were 0.0074, 6.5, 49, 38, 0.0055, 0.92, >100, 1.4, >100, 2.4, 70, 39 and 17, respectively.

Table 1. ^{13}C- and ^{1}H-NMR Chemical Shifts (δ) for Cedronolactones A (1) - D (4), Simalikalactone D (5), and Ailanquassin A (12).[a,b]

	cedronolactone A (1)		cedronolactone B (2)		cedronolactone C (3)		cedronolactone D (4)		simalikalactone D (5)		ailanquassin A (12)	
position	δC	δH mult. (J /Hz)	δC	δH mult. (J /Hz)	δC	δH mult. (J /Hz)	δC	δH mult. (J /Hz)	δC	δH mult. (J /Hz)	δC	δH mult. (J /Hz)
1	82.9 d	4.15 s					84.3 d	4.22 d (2.0)	81.1 d	4.12 s		
2	198.4 s		172.5 s		172.5 s		198.7 s		196.6 s		172.6 s	
3	125.1 d	6.13 s	120.5 d	5.88 s	120.6 d	5.89 t (1.4)	124.7 d	6.13 q (1.3)	123.2 d	6.12 s	119.0 d	5.93 s
4	162.9 s		168.2 s		168.0 s		164.8 s		161.1 s		169.9 s	
5	43.7 d	2.91 brd (12)	91.8 d	4.85 s	91.7 d	4.87 s	43.6 d	3.47 brd (12)	42.0 d	2.90 brd (12)	92.2 d	4.97 brs
6	28.3 t	2.21 dt (2.4, 14.7)	47.1 t	2.54 d (15.7)	46.1 t	2.51 d (15.7)	31.3 t	2.13 dt (2.2, 13.4)	26.5 t	2.20 dt (2.7, 14.6)	46.5 t	2.9 3d (16.1)
		1.72 dt (14.7, 2.4)		2.31 dd (15.7, 5.7)		2.27 dd (15.7, 5.2)		1.67 dt (13.4, 2.2)		1.71 dt (14.6, 2.7)		2.32 dd (16.1, 4.7)
7	84.3 d	4.87 t (2.4)	83.7 d	4.72 d (5.7)	83.4 d	4.76 d (5.2)	72.8 d	4.27 brs	82.4 d	4.87 t (2.7)	80.5 d	4.66 d (4.7)
8	46.6 s		55.6 s		56.7 s		50.3 s		46.5 s		57.0 s	
9	43.0 d	2.72 d (4.4)	45.0 d	3.38 s	46.3 d	3.49 s	44.6 d	2.54 d (4.1)	41.3 d	2.71 d (4.5)	44.1 d	3.34 s
10	48.3 s		46.1 s		45.8 s		48.7 s		44.8 s		46.5 s	
11	75.5 d	5.41 t (4.4)	111.7 s		112.0 s		72.9 d	5.53 dd (2.2, 4.1)	78.3 d	5.40 t (4.5)	111.3 s	
12	80.1 d	4.31 d (4.4)	80.2 d	3.97 t (3.6)	81.0 d	4.12 d (3.8)	87.0 d	4.67 t (2.2)	73.7 d	4.31 d (4.5)	83.8 d	3.95 t (4.3)
13	81.2 s		33.3 d	2.35 m	34.6 d	2.64 m	76.9 s		79.5 s		33.4 d	2.38 m
14	53.1 d	2.83 brd (13)	38.9 d	2.11 m	47.1 d	2.52 dd (10.4, 6.1)	58.1 d	2.84 d (2.5)	51.4 d	2.81 brd (13)	38.6 d	2.19 dd (12.5, 6.3)
15	68.9 d	4.95 d (6.2)	30.5 t	3.26 dd (18.2, 13.1)	68.5 d	5.38 d (10.4)	66.5 d	5.95 s	67.1 d	4.93 d (12.7)	30.5 t	3.28 dd (18.4, 12.5)
				2.76 dd (18.2, 5.8)								2.82 dd (18.4, 6.3)
16	168.7 s		169.6 s		173.9 s		173.7 s		166.8 s		170.0 s	
18	22.1 q	1.72 s	16.5 q	2.05 s	16.5 q	2.01 s	22.5 q	1.76 s	22.2 q	1.71 s	16.1 q	2.49 s
19	11.4 q	1.41 s	20.8 q	1.59 s	20.6 q	1.58 s	11.9 q	1.50 s	10.0 q	1.41 s	18.4 q	1.51 s
20	72.3 t	5.01 d (7.4)	72.1 t	3.94 d (8.7)	72.3 t	3.92 s	74.7 t	4.97 d (7.8)	70.5 t	5.00 d (7.4)	72.0 t	3.94 d (8.7)
		3.72 d (7.4)		3.83 d (8.7)		3.92 s		3.74 d (7.8)		3.72 d (7.4)		3.82 d (8.7)
21	23.9 q	1.79 s	12.9 q	1.08 d (7.2)	15.9 q	1.69 d (7.3)	22.8 q	1.78 s	15.0 q	1.78 s	12.7 q	1.11 d (7.2)
1'	171.6 s								173.3 s			
2'	43.4 t	2.41 dd (7.5, 4.8)							39.7 d	2.60 m		
3'	25.9 d	2.27 m							26.5 t	1.88 m		
4'	22.4 q	1.00 d (6.3)							20.3 q	1.04 t (7.4)		
5'	22.4 q	1.01 d (6.3)							9.6 q	1.26 d (7.0)		

[a] Measurements were performed in pyridine-d_5 at 400 MHz for ^{1}H and 100 MHz for ^{13}C. [b] ^{13}C Multiplicities were established by DEPT pulse sequences.

Experimental Section

General Experimental Procedures. Melting points are uncorrected. UV spectra were taken on a Hitachi 557 spectrophotometer. IR spectra were run on a Perkin-Elmer 1710 or a JASCO A-302 spectrophotometer. ^{1}H-, ^{13}C- and 2D (COSY, NOESY, HMBC and HMQC) NMR spectra were measured by a Bruker AM 400 or a AM 500 spectrometer. ^{1}H-NMR chemical shifts are referenced in pyridine-d_5 to residual C_5D_4HN (7.21 ppm); ^{13}C-NMR chemical shifts are referenced to the solvent (135.5 ppm). Mass spectra were taken with a VG AutoSpec E or a Finnigan MAT TSQ-700 spectrometer. Preparative HPLC was carried out on a Shimadzu HPLC system using a Wakosil-II $5C_{18}$ HG Prep (20 × 250 mm) column with UV detector. Medium-pressure liquid chromatography (MPLC) was carried out using a Kusano C.I.G. system (Kusano, Tokyo, Japan).

Plant Material. The wood of *Simaba cedron* Planchon (Simaroubaceae) was purchased at São Paulo, Brazil in 1991. The botanical identification was made by Dr. S. De M. Alves. A voucher specimen has been deposited in the herbarium of Tokyo University of Pharmacy & Life Science.

Extraction and Isolation. The wood of *S. cedron* (2.0 kg) was extracted with MeOH (3 × 4 L). The MeOH extract [120 g, IC_{50} value (μg/mL) against P388 cells: 0.7] was partitioned between $CHCl_3$ and H_2O, and then between *n*-BuOH and H_2O. The $CHCl_3$-soluble fraction (30 g, IC_{50} 0.22 μg/mL) was subjected to column chromatography over silica gel using a $CHCl_3$–MeOH (1:0–0:1) gradient system to give eight fractions. The fourth fraction (IC_{50} <0.1 μg/mL) was further applied to MPLC (silica gel) using *n*-hexane–EtOAc–MeOH (5:3:1) as solvent system and then to HPLC (ODS silica gel, with mixture of MeOH–H_2O and MeCN–H_2O as solvent systems) to give cedronolactone A (**1**, 79 mg) and simalikalactone D (**5**, 93 mg). The fifth fraction (IC_{50} 0.17 μg/mL) was subjected to MPLC (silica gel) using *n*-hexane–EtOAc–MeOH (5:4:1) and then to HPLC (ODS silica gel) using either a MeOH–H_2O or a MeCN–H_2O (20:1–1:1) gradient system to give cedronolactone B (**2**, 25 mg), chaparrinone (**6**, 134 mg), glaucarubolone (**8**, 186 mg), and ailanquassin A (**12**, 40 mg). Repeated MPLC (ODS silica gel) of the sixth fraction using a MeOH–H_2O gradient system (IC_{50} 4.0 μg/mL) furnished guanepolide (**11**, 7.5 mg).

The *n*-BuOH soluble fraction (41 g, IC_{50} 6 μg/mL) was applied to HP-20 column chromatography using a H_2O–MeOH (1:0–0:1) gradient system to give seven fractions (A–G). Fraction C (IC_{50} 21 μg/mL) was purified by MPLC (silica gel) using $CHCl_3$–MeOH (9:1) and then HPLC (ODS silica gel), using H_2O–MeOH (17:3), to give cedronolactone C (**3**, 257 mg), polyandrol (**13**, 261 mg), and samaderine Z (**10**, 375 mg). Fraction D (IC_{50} 16 μg/mL) was crystallized from MeOH to give a crude crystal, which was then subjected to HPLC (ODS silica gel) to afford chaparrin (**7**, 78 mg), glaucarubolone (**8**, 1.043 g), and glaucarubol (**9**, 1.139 g). Cedronolactone D (**4**, 10 mg) was obtained from the mother liquid by using HPLC (ODS silica gel).

Cedronolactone A (1): Colorless needles, mp 185–188 °C; $[\alpha]^{25}_D$ –40° (*c* 0.11, pyridine); UV (EtOH) λ_{max} (log ε) 240 (4.02) nm; IR (KBr) ν_{max} 3436, 1752, 1666, 1377, 1346, 1262, 1158, 1118 cm^{-1}; ^1H- and ^{13}C-NMR data, see Table 1; EIMS *m/z* 478 $[M]^+$ (13), 3460 (7), 376 (6), 358 (19), 340 (17), 301 (15), 255 (18), 236 (22), 195 (36), 152 (22), 135 (24), 111 (29), 84 (51), 55 (100); HREIMS *m/z* 4782219 (calcd. for $C_{25}H_{34}O_9$, 478.2203).

Cedronolactone B (2): Colorless needles, mp 194–196 °C. $[\alpha]^{25}_{D}$ –38° (*c* 0.19, pyridine); UV (MeOH) λ_{max} (log ε) 213 (3.98) nm; IR (KBr) ν_{max} 3392, 1742, 1709, 1637, 1322, 1256, 1194, 1119 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; EIMS *m/z* 364 $[M]^{+}$ (49), 346 (11), 333 (8), 318 (20), 305 (65), 292 (13), 267 (29), 231 (17), 207 (23), 191 (25), 173 (27), 145 (33), 125 (41), 97 (82), 68 (100), 53 (91); HREIMS *m/z* 364.1513 (calcd. for $C_{19}H_{24}O_7$, 364.1522).

Cedronolactone C (3): Colorless needles, mp 99–105 °C. $[\alpha]^{25}_{D}$ +75° (*c* 0.44, pyridine); UV (MeOH) λ_{max} (log ε) 213 (4.04) nm; IR (KBr) ν_{max} 3510, 1736, 1631, 1316, 1231, 1191, 1104 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; EIMS *m/z* 380 $[M]^{+}$ (6), 362 (22), 321 (37), 305 (12), 265 (18), 217 (41), 189 (33), 145 (41), 137 (100), 98 (46), 97 (83), 77 (44); HREIMS *m/z* 380.1468 (calcd. for $C_{19}H_{24}O_8$, 380.1471).

Cedronolactone D (4): Amorphous solid. $[\alpha]^{25}_{D}$ –55° (*c* 0.10, pyridine); UV (MeOH) λ_{max} (log ε) 241 (3.86) nm; IR (KBr) ν_{max} 3539, 3400, 1724, 1697, 1677, 1262, 1113 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; EIMS *m/z* 394 $[M]^{+}$ (53), 376 (57), 343 (15), 279 (16), 271 (24), 253 (43), 225 (57), 207 (100), 169 (69), 149 (63), 105 (52), 91 (83), 69 (61); HREIMS *m/z* 394.1621 (calcd. for $C_{20}H_{26}O_8$, 394.1628).

Selective Epimerization of 12. A solution of **12** (22.2 mg) in pyridine (0.5 mL) was stirred at 150 °C for 24 h under an Ar atmosphere. The solution was evaporated *in vacuo*. The residue was separated by HPLC (ODS silica gel) using H_2O–MeOH (25:3) to give **2** (6.7 mg) and recovered **12** (11.1 mg).

Selective Epimerization of 13. A solution of **13** (20.5 mg) in pyridine (0.5 mL) was stirred at 150 °C for 24 h under an Ar atmosphere. The solution was evaporated *in vacuo*. The residue was separated by HPLC (ODS silica gel) using H_2O–MeOH (25:2) to give **3** (4.5 mg) and recovered **13** (12.0 mg).

Cytotoxic Activity against P388 Cells.[20,21] An MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay was performed in 96-well plates. The assay is based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to give a blue formazan product which can be measured spectrophotometrically. Murine P388 leukemia cells (3 x 10^4 cell/mL) were inoculated in each well with 100 μL/mL of RPMI-1640 medium (Nissui Pharmaceutical Company, Ltd., Tokyo, Japan) supplemented with 5% fetal calf serum (Mitsubishi Chemical Industry Co., Ltd., Tokyo, Japan) and kanamycin (100

μg/mL) at 37 °C in a humidified atmosphere of 5% CO_2. Various drug concentrations (10 mL) were added to the cultures at day 1 after transplantation. At day 3, 20 μL of MTT solution (5 mg/mL) per well was added to each cultured medium. After a further 4 h of incubation, 100 μL of 10% sodium dodecylsulfate–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 microplate reader (Tosoh MPR-A4i) at two wavelengths (550 and 700 nm). In all these experiments, three replicate wells were used to determine each data point.

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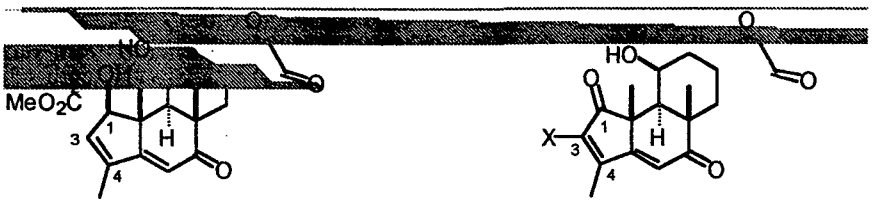
Eurycolactones A–C, novel quassinoids from *Eurycoma longifolia*

Abstract. Three novel quassinoids, eurycolactones A–C (**1**–**3**), were isolated from the roots of *Eurycoma longifolia* Jack. Their structures were elucidated by interpretation of the spectroscopic data.

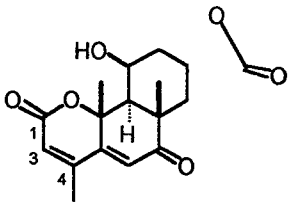
The Simaroubaceae family is a large family comprising 30 genera and 200 species, of which 8 genera and 10 species are found in Malaysia.[1] *Eurycoma longifolia* Jack is a Malaysian plant of this family known for its diverse biological activities, such as antimalarial,[2] antiulcer,[3] antipyretic[4] and cytotoxic[5] activities. In our present study, from this plant, we isolated three novel C_{19} and C_{18} quassinoids, eurycolactones A–C (**1**–**3**),[6] having unique structural features along with several known quassinoids including 5,6-dehydroeurycomalactone (**4**)[7] and laurycolactone B (**5**)[8] and elucidated the structures of these novel quassinoids.

The methanol extract obtained from the dried roots of *E. longifolia* (50 kg) was partitioned between chloroform and water. The chloroform-soluble portion was subjected to silica gel and Diaion HP-20 column chromatography, and finally to reversed-phase HPLC to afford eurycolactones A–C (**1**–**3**).

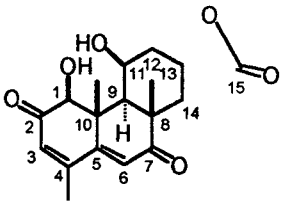


1: Eurycolactone A

2: Eurycolactone B (X = Cl)
5: Laurycolactone B (X = H)

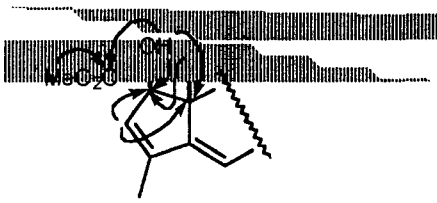


3: Eurycolactone C



4: 5,6-Dehydroeurycomalactone

Fig. 1.



HMBC: H → C

Fig. 2. Selected HMBC correlations for the A ring moiety of **1**

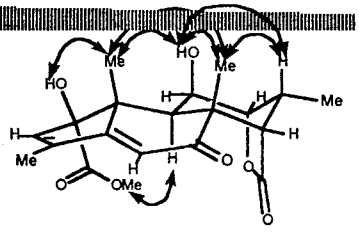
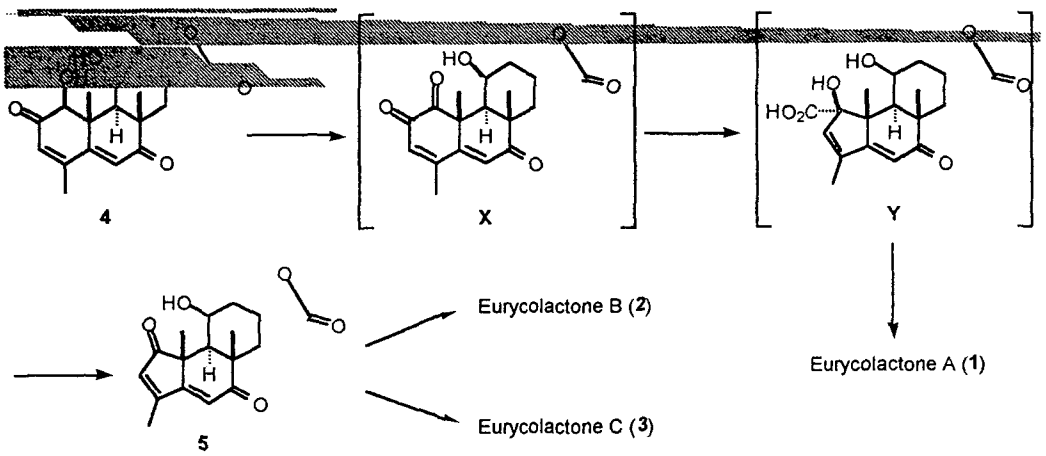


Fig. 3. Key NOESY correlations for **1**

Eurycolactone A (**1**)[9] (13.3 mg, 2.7 × 10^{-5}% yield), $[\alpha]_D^{26}$ +141° (*c* 0.13, MeOH), was obtained as colorless plates, mp 228–233 °C (decomp.). The molecular formula was established as $C_{20}H_{24}O_7$ by HREIMS (*m/z* 376.1522, M^+, Δ ±0 mmu). Comparison of the ^1H and ^{13}C NMR spectra of **1** (Tables 1 and 2) with those of **5**[10] indicated that **1** had the same B and C rings as **5** and that, accordingly, the structural differences between the two quassinoids resided only in the A ring. The NMR spectra showed the presence of an extra hydroxyl group (δ 7.78) and a methoxycarbonyl group (δ_H 3.79; δ_C 52.68 and 174.51) in **1** and the HMBC correlations observed between 1-OH and C-1, C-2 and C-10 (Fig. 2) implied that the two groups were both attached to C-1. The stereochemistry of C-1 was confirmed by the analysis of its NOESY spectrum. Correlations were observed between 1-OH and 10-Me, and between 2-OMe and 9-H, which indicated that the

hydroxyl group and the methoxycarbonyl group were in the β- and α-orientations, respectively (Fig. 3). Thus, eurycolactone A (**1**) was shown to be a quassinoid of a novel carbon framework as shown in Figure 1.

Eurycolactone B (**2**)[9] (13.3 mg, 2.7 × 10^{-5}% yield), $[\alpha]_D^{26}$ +82° (*c* 0.13, MeOH), was obtained as pale yellow prisms, mp 259 °C. Its molecular formula was determined as $C_{18}H_{19}ClO_5$ by HREIMS (*m/z* 350.0902, M^+, Δ +1.9 mmu). The presence of a chlorine atom was also demonstrated by an isotope peak, $[M+2]^+$, at *m/z* 352 of one third of the intensity of the molecular ion peak (M^+). Its ^1H and ^{13}C NMR spectra were quite similar to those of **5**[10] except that **2** had no 3-H resonance, and that the C-3 resonance of **2** was observed as a quaternary carbon at δ 136.43, which was 3.07 ppm downfield from the corresponding resonance of **5**, as expected for a chlorine-bearing carbon resonance. On the basis of these observations and the molecular formula demonstrated by HREIMS, we determined that eurycolactone B (**2**) has a structure in which 3-H of **5** is substituted by a chlorine atom, as shown in Figure 1. Compound **2** is the first halogenated quassinoid separated from plant sources.



Scheme 1. A possible biogenetic pathway for eurycolactones A (**1**–**3**) and laurycolactone B (**5**) from 5,6-dehydroeurycomalactone (**4**)

Eurycolactone C (**3**)[9] (5.9 mg, 1.2 × 10^{-5}% yield), $[\alpha]_D^{26}$ −119° (*c* 0.06, MeOH), was obtained as colorless plates, mp 259–263 °C (decomp.). Its molecular formula was determined to be $C_{18}H_{20}O_6$ by HREIMS (*m/z* 332.1251, M^+, Δ +0.9 mmu), which showed that **3** had the molecular formula of **5** with one more oxygen atom. The ^1H NMR spectra of **3** and **5** were very similar, and in the ^{13}C NMR spectrum the upfield shift (Δδ 43.49) of the C-1 resonance relative to that of **5**[10] and

the resonance of C-10 at δ 81.38 suggested that a lactone linkage existed between C-1 and C-10 of **3**. From these data and NOESY spectra, eurycolactone C (**3**) was shown to be a quassinoid of an unusual structure having lactone A ring as shown in Figure 1.

Table 1 ^{1}H NMR chemical shifts assignments for eurycolactones A–C (**1**–**3**) in C_5D_5N[a]

position	1	2	3
3	6.18 (d, 1.4)	—	6.17 (br s)
6	6.04 (s)	6.13 (s)	6.19 (s)
9	2.79 (d, 2.9)	2.63 (d, 3.3)	2.88 (d, 3.2)
11	5.05 (m)	5.47 (m)	5.13 (m)
12	4.41 (dd, 5.0, 1.1)	4.48 (dd, 4.9, 1.0)	4.50 (br d, 4.2)
13	3.34 (q, 7.0)	3.32 (q, 7.0)	3.27 (q, 7.0)
14	3.43 (d, 1.1)	3.34 (d, 1.0)	3.30 (d, 0.9)
4-Me	1.85 (d, 1.4)	2.04 (s)	1.94 (d, 1.3)
8-Me	1.83 (s)	1.77 (s)	1.60 (s)
10-Me	2.12 (s)	1.85 (s)	2.08 (s)
13-Me	1.02 (d, 7.0)	1.06 (d, 7.0)	1.07 (d, 7.0)
1-OH	7.78 (s)	—	—
11-OH	7.62 (d, 5.6)	7.95 (d, 5.9)	7.94 (d, 5.9)
2-OMe	3.79 (s)	—	—

[a] The spectra were obtained at 500 MHz. Chemical shifts are reported in ppm relative to C_5D_4HN resonance at 7.21 ppm. *J*-Values are given in parentheses in Hz.

Table 2 ^{13}C NMR chemical shifts assignments for eurycolactones A–C (**1**–**3**) in C_5D_5N[a]

position	1	2	3
1	90.29	197.50	161.70
2	174.51	—	—
3	142.01	136.43	122.06
4	142.32	158.17	148.34
5	173.52	162.86	153.63
6	114.02	116.44	123.72
7	198.48	197.80	197.42
8	47.38	48.47	47.69
9	41.93	40.51	46.41
10	56.66	48.93	81.38
11	68.27	67.10	65.40
12	84.50	84.72	84.20
13	32.73	32.49	32.24
14	54.16	53.67	52.79
15	177.49	176.97	176.64
4-Me	12.31	11.64	17.96
8-Me	23.25	23.13	20.91
10-Me	21.26	21.36	24.27
13-Me	17.04	16.87	16.68
2-OMe	52.68	—	—

[a] The spectra were obtained at 125 MHz. Chemical shifts are reported in ppm relative to the solvent resonance at 135.5 ppm.

In the present study, compounds **1–5** whose structures were very similar to each other were isolated from the same plant source suggesting some biosynthetic relations among them. Of particular interest is the isolation of eurycolactone A (**1**) which has apparently an intermediate key structure between C_{18} quassinoids and C_{19} quassinoids. Therefore, a possible biogenetic pathway for laurycolactone B (**5**) and eurycolactones A–C (**1–3**) from 5,6-dehydroeurycomalactone (**4**) is proposed in Scheme 1. Compound **4** is oxidized to triketone **X**, which is then converted into intermediate **Y** via a benzilic acid-type rearrangement, which via oxidative decarboxylation produces laurycolactone B (**5**). Methylation of **Y** produces eurycolactone A (**1**), chlorination of **5** affords eurycolactone B (**2**), and oxidation of **5** involving a Baeyer-Villiger-type reaction produces eurycolactone C (**3**).

References and notes

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9. *Spectral data* **1**: UV (MeOH) λ_{max} (log ε): 284 (4.16) nm; IR (film) ν_{max}: 3471, 1775, 1730, 1650, 1618 cm^{-1}. **2**: UV (MeOH) λ_{max} (log ε): 222 (3.83), 297 (4.18) nm; IR (film) ν_{max}: 3492, 1777, 1728, 1667 cm^{-1}. Selected NOESY correlations: 4-Me/6-H, 8-Me/10-Me, 8-Me/11-OH, 8-Me/13-H, 8-Me/14-H, 10-Me/11-OH. **3**: UV (MeOH) λ_{max} (log ε): 279

(4.15) nm; IR (film) ν_{max}: 3465, 1779, 1714, 1677 cm^{-1}. Selected NOESY correlations: 3-H/4-Me, 4-Me/6-H, 8-Me/10-Me, 8-Me/11-OH, 8-Me/13-H, 8-Me/14-H, 10-Me/11-OH.

10. **5**: ^1H NMR (500 MHz, C_5D_5N, ref: C_5D_4HN = 7.21 ppm, *J*/Hz) δ 1.06 (3H, d, *J* 7.0, 13-Me), 1.78 (3H, s, 8-Me), 1.83 (3H, s, 10-Me), 1.98 (3H, s, 4-Me), 2.54 (1H, d, *J* 3.3, 9-H), 3.34 (1H q, *J* 7.0, 13-H), 3.35 (1H, s, 14-H), 4.49 (1H, d, *J* 4.8, 12-H), 5.54 (1H, m, 11-H), 6.04 (1H, s, 6-H), 6.07 (1H, br s, 3-H), 7.85 (1H, d, *J* 6.0, 11-OH); ^{13}C NMR (125 MHz, C_5D_5N, ref: C_5D_5N = 135.5 ppm) δ 13.39 (4-Me), 16.93 (13-Me), 21.38 (10-Me), 23.12 (8-Me), 32.53 (C-13), 41.07 (C-9), 48.14 (C-8), 49.42 (C-10), 53.72 (C-14), 67.20 (C-11), 84.77 (C-12), 116.04 (C-6), 133.36 (C-3), 164.51 (C-4), 166.65 (C-5), 177.16 (C-15), 198.40 (C-7), 205.19 (C-1).