สิทธิบัตร

1) ฝ่ายวิชาการ สกว. ได้ให้ความเห็นชอบในการจดสิทธิบัตรผลงานวิจัยเรื่อง "บราสสิโนสเตียรอยด์อนาลอกซึ่งแสดงฤทธิ์ควบคุมการเจริญเติบโตของพืช" (Brassinosteroid analogues with plant growth regulating activity) โดย สุรีย์พร หอมวิเศษวงศา, Yasutomo Takeuchi, อภิชาต สุขสำราญ ขณะนี้กำลังร่างรายละเอียดการประดิษฐ์ของสิทธิบัตรอยู่

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Antimycobacterial Activity of Prenylated Xanthones from the Fruits of *Garcinia mangostana*

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Prenylated xanthones, isolated from the fruit hulls and the edible arils and seeds of *Garcinia mangostana*, were tested for their antituberculosis potential. α- and β-Mangostins and garcinone B exhibited strong inhibitory effect against *Mycobacterium tuberculosis* with the minimum inhibitory concentration (MIC) value of 6.25 μg/ml. Tri- and tetra-oxygenated xanthones with di-C_5 units or with a C_5 and a modified C_5 groups are essential for high activities. Substitution in the A and C rings has been shown to modify the bioactivity of the compounds.

Key words antituberculosis; prenylated xanthone; *Garcinia mangostana*; *Mycobacterium tuberculosis*; structure–activity relationship

Plants have been used worldwide in traditional medicines for the treatment of diseases. It is estimated that even today approximately two-thirds to three-quarters of the world's population rely only on medicinal plants as their primary source of medicines. Mangosteen, *Garcinia mangostana* L. (Clusiaceae), is a tree fairly widespread in Southeast Asian countries, known for its medicinal properties. The edible fruit of this plant is considered to be one of the best of all tropical fruits. The fruit hulls have been in use in Thai folk medicine for the treatment of skin infections, wounds and diarrhea.[1] Phytochemical studies have shown that this plant species are rich in a variety of prenylated xantones[2,3] and the constituents have demonstrated a number of bioactivities.[4—11] Tuberculosis continues to be an enormous global concern as it infects millions of people annually and the search for new drug leads is an urgent need due to the emergence of drug resistant strains of mycobacterial. A tuberculostatic effect has been noted in natural and synthetic non-prenylated xanthones,[12—14] and quantitative structure–activity relationships investigations have established correlations between ^{13}C-NMR chemical shifts, lipophilicity and molar refractivities of the substituents of various synthetic non-prenylated xanthones and tuberculosis inhibition.[15,16] However no study on the antimycobacterial potential of prenylated xanthones has been described. We have previously collected a number of prenylated xanthones isolated from the fruit hulls of this plant.[17] We report here on the inhibitory activity of the prenylated xanthones obtained from the fruits of *G. mangostana* against *Mycobacterium tuberculosis* in *in vitro* experiments.

Prenylated xanthones **1**, **2**, **5**, **6**, **9**—**11**, **14**, and **15** were previously isolated from the green fruit hulls of *G. mangostana*.[17] In order to obtain additional compounds for the testing, four known xanthones γ-mangostin (**3**),[11] garcinone D (**4**),[18,19] mangostanin (**8**)[20] and 1,7-dihydroxy-2-(3-methylbut-2-enyl)-3-methoxyxanthone (**12**)[1] were isolated from a larger quantity of the MeOH extract of the fresh green fruit hulls. Compounds **7**,[21] **13**,[1] and more of compound **1**[17] were obtained from the MeOH extract of the pulverized fresh arils and seeds (see Experimental).

The above prenylated xanthones **1**—**15**, the structures of which are shown in Chart 1, were examined in the present screening test. The inhibitory activity of **1**—**15** against *Mycobacterium tuberculosis* H_{37}Ra strain were determined using the Microplate Alamar Blue Assay (MABA).[22] The minimum inhibitory concentration (MIC) data (Table 1) of the 15 xanthones suggested that, for a moderate to high antimycobacterial activity, the xanthones nucleus should contain tri- or tetra-oxygen functions with either di-C_5 units or with a C_5 and a modified C_5 groups in rings A and C. Among these, 1,3,6,7-tetraoxygenated xanthones bearing the C_5 units at C-2 and C-8 in α-mangostin (**1**), the major constituent, β-mangostin (**2**) and garcinone B (**6**) exhibited the most potent activities with the same MIC value of 6.25 μg/ml. γ-Mangostin (**3**), the second major constituent bearing C-3 and C-7 hydroxyls, exhibited lower inhibitory activity, suggesting that methylation of the 3-hydroxyl as well as the 7-hydroxyl groups resulted in increasing activity. A structure–activity comparison of **1** with **4** and mangostenol (**5**) revealed that modifications of the C_5 units in either C-8 or C-2 altered the activity. Furthermore, cyclization of the C_5 group at C-2 position resulted in decreasing inhibitory activity as exemplified by **7**, **8** and mangostanol (**9**). It is of interest to note that increment in polarity of the C_5 side chain reduced the activity, and addition of the third C_5 moiety in mangostenone A (**10**) and tovophyllin B (**11**), with respect to **6** and **7**, affected the inhibitory activity. In the case of 1,3,7-trioxygenated compounds, the inactivity of **12**, the xanthone with only one C_5 group located at C-2, compared with the moderately active demethylcalabaxanthone (**13**), indicated the essential of the di-C_5 side chains in the nucleus. For 1,3,5-trioxygenated xanthones, the more potent activity of trapezifolixanthone (**14**) than that of mangostinone (**15**) further supported this fact.

Experimental

General Procedures ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance 300 FT-NMR spectrometer, operating at 300 MHz (^{1}H) and 75 MHz (^{13}C). Column chromatography and TLC were carried out using Merck silica gel 60 (>230 mesh) and precoated silica gel 60 F_{254} plates, respectively. Plates of silica gel PF_{254}, thickness 1.25 mm, were utilized for preparative TLC. Spots on TLC were visualized under UV light and by spraying with anisaldehyde–H_2SO_4 reagent followed by heating.

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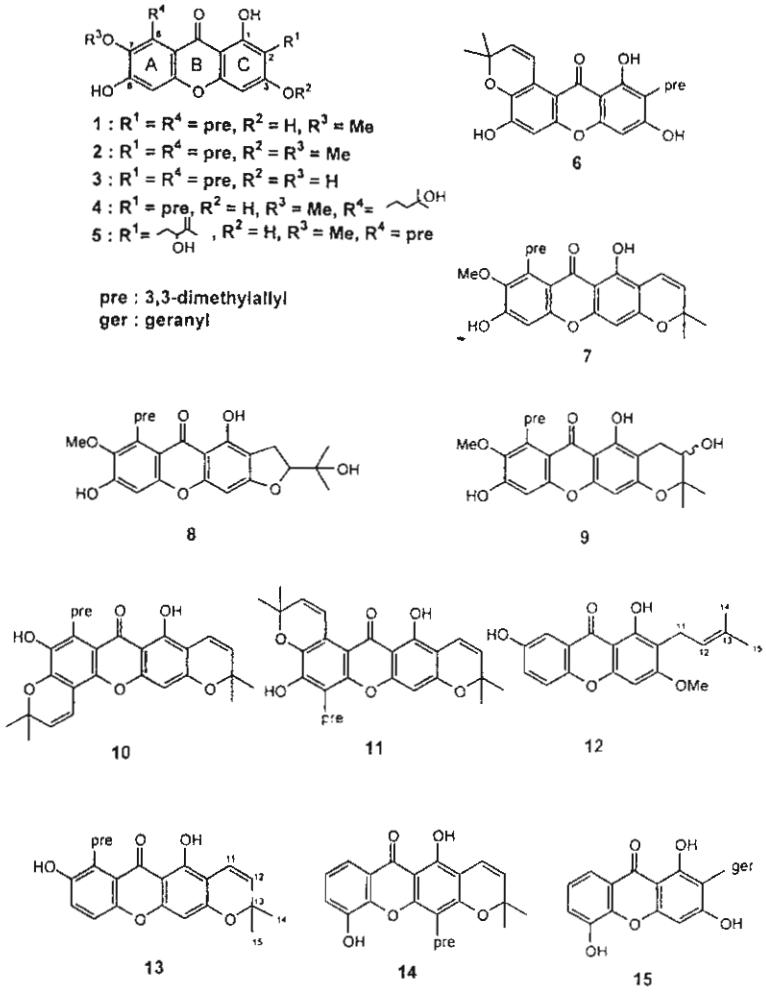

Chart 1. Structures of 1—15

Table 1. MIC Value (μg/ml) for Compounds 1—15 against *Mycobacterium tuberculosis*

Compound	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
MIC	6.25	6.25	25	25	100	6.25	12.5	25	200	25	25	Inactive[a]	12.5	12.5	200

a) Inactive at >200 μg/ml.

Plant Material The fruits of *G. mangostana* were collected from Bahnkai District, Chanthaburi Province, Thailand, in April 1999. A voucher specimen [voucher #0032(RU)] of this plant is deposited at the Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand.

Extraction and Isolation On the larger scale investigation of the MeOH extract obtained from the fresh green fruit hulls of *G. mangostana*, four known xanthones γ-mangostin (**3**,[11] 100 mg), garcinone D (**4**,[18,19] 10 mg), mangostanin (**8**,[20] 7 mg) and 1,7-dihydroxy-2-(methylbut-2-enyl)-3-methoxyxanthone (**12**,[1] 2 mg) were further identified, in addition to those obtained previously.[15] Pulverized, fresh arils and seeds (4.7 kg) were extracted throroughly with MeOH and evaporation of the solvent gave crude extract (487.0 g). The crude extract was partitioned between $CHCl_3$ and H_2O to afford $CHCl_3$ extract (11.3 g). Repeated column chromatography of the $CHCl_3$ extract using $CHCl_3$, acetone and MeOH yielded demethylcalabaxanthone (**13**,[1] 80 mg), **7**[21] (180 mg) and α-mangostin (**1**,[17] 1.3 g) in order of polarity. All compounds were identified by comparison of their spectroscopic data (NMR and MS) with those reported in the literature. ^{13}C-NMR data of **12** and **13**, which have not previously been published, are also collected in this paper.

1,7-Dihydroxy-2-(3-methylbut-2-enyl)-3-methoxyxanthone (**12**): ^{13}C-NMR (acetone-d_6) δ: 181.5 (s, 9-C), 165.3 (s, 3-C), 160.0 (s, 1-C), 157.2 (s, 4a-C), 154.8 (s, 7-C), 150.7 (s, 8a-C), 131.7 (s, 13-C), 125.1 (d, 6-C), 123.0 (d, 12-C), 121.8 (s, 10a-C), 119.7 (d, 5-C), 111.8 (s, 2-C), 109.2 (d, 8-C), 104.2 (s, 9a-C), 90.6 (d, 4-C), 56.6 (q, OMe), 25.8 (q, 14-C), 21.8 (t, 11-C), 17.8 (q, 15-C)

1,7-Dihydroxy-8-(3-methylbut-2-enyl)-6′,6′-dimethylpyrano(2′,3′:3,2)-xanthone (**13**): ^{13}C-NMR ($CDCl_3$+CD_3OD) δ: 183.4 (s, 9-C), 160.1 (s, 3-C), 157.6 (s, 1-C), 156.6 (s, 4a-C), 151.5 (s, 10a-C), 151.1 (s, 7-C), 132.7 (s, 8-C), 128.2 (s, 18-C), 127.2 (d, 12-C), 123.0 (d, 6-C), 122.7 (d, 17-C), 118.9 (s, 8a-C), 116.1 (d, 5-C), 115.7 (d, 11-C), 104.1 (s, 2-C), 94.4 (s, 9a-C), 94.1 (d, 4-C), 78.0 (s, 13-C), 28.3 (q, 14-C, 15-C), 25.9 and 18.1 (2q, 19-C, 20-C), 25.7 (t, 16-C).

Bioassay Procedure The antimycobacterial activity was assessed against *M. tuberculosis* $H_{37}Ra$ using the Microplate Alamar Blue Assay.[22]

Briefly, initial candidate compound dilutions were prepared in dimethyl sulfoxide (DMSO), and subsequent twofold dilutions were performed in 0.1 ml of 7H9GC medium in the microculture plates. 100 μl of 5×10^4 CFU/ml of *M. tuberculosis* in 7H9GC-Tween was added to each well of 96 well microculture plates containing of test compound. Plates were incubated at 37 °C for 7 d. To three control wells which contained drug and medium, bacteria and medium, and medium only, the Alarmar Blue dye solution (20 μl of Alarmar Blue solution and 12.5 μl of 20% Tween) was added daily until a color change from blue to pink occurred, at which time the dye was added to all remaining wells. Plates were incubated at 37 °C, and results were recorded at 24 h post-dye addition. Fluorescence was measured in a Cytofluoro Series 4000 Fluorescence Multi-Well Plate Reader (Per-Septive Biosystems, Framingham, MA, U.S.A.) in bottom-reading mode with excitation at 530 nm and emission at 590 nm. Percent inhibition was defined 1−(test well FU/mean FU of triplicate control wells)×100. The lowest drug concentration effecting an inhibition of ≥90% was considered the MIC. Experiments were usually completed within 10 d. Standard drugs rifampicin, isoniazid and kanamycin sulfate showed MIC of 0.003—0.0047, 0.025—0.05 and 1.25—2.5 μg/ml, respectively.

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A Bioactive Triterpenoid and Vulpinic Acid Derivatives from the Mushroom *Scleroderma citrinum*

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Abstract

A new lanostane-type triterpenoid, (20*S*,22*S*,23*E*)-22-*O*-acetyl-25-hydroxylanosta-8,23(*E*)-dien-3-one (**1**), isolated as a new natural product for the first time, methyl 4,4′-dimethoxyvulpinate (**2**), together with the known compound 4,4′-dimethoxyvulpinic acid (**3**) were isolated from the mushroom *Scleroderma citrinum*. Their structures were determined using spectroscopic and chemical methods, and an X-ray analysis was performed to confirm structure of **1**. Compound **1** exhibited significant antiviral activity against *Herpes simplex* type 1. Compound **3** and two of its derivatives, the dibromo derivative **5** and acetate derivative **6**, exhibited activity towards *Mycobacterium tuberculosis*. In addition, **5** and **6** also showed cytotoxicity against the NCI-H187 cell line.

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Scleroderma citrinum Pers. or "earth ball" or "poison puffball" (Gasteromycetes) has been reported as a poisonous fungus [1]. However, its phytochemistry has not been reported. In our search for bioactive constituents from macrofungi and mushrooms in Thailand, the methanol extract of *S. citrinum* showed potential antiviral activity against *Herpes simplex* type 1 (IC_{50} = 15 μg/mL) and weak activity against *Mycobacterium tuberculosis* H37Ra (MIC = 100 μg/mL). Bioassay-guided fractionation of the MeOH extract led to the isolation of a new lanostane-type triterpenoid **1** as well as two known vulpinic acid derivatives, methyl 4,4′-dimethoxy vulpinate (**2**) [2], [3] and 4,4′-dimethoxyvulpinic acid (**3**) [4], [5]. Although **2** has been previously reported as a permethylation product of atromentic acid (**7**) [2], this is the first report as a new compound from natural source. In addition, the dibromo derivatives of **2**, methyl 3,3′-dibromo-4,4′-dimethoxy vulpinate (**4**) and of **3**, 3,3′-dibromo-4,4′-dimethoxyvulpinic acid (**5**) as well as the acetate derivative of **3**, acetyl 4,4′-dimethoxyvulpinate (**6**), were prepared for chemical investigation as well as biological evaluation.

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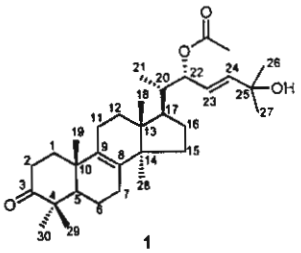

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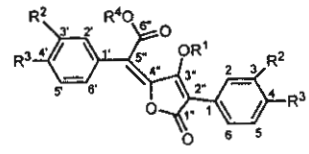

	R^1	R^2	R^3	R^4
2	Me	H	OMe	Me
3	H	H	OMe	Me
4	Me	Br	OMe	Me
5	H	Br	OMe	Me
6	Ac	H	OMe	Me
7	H	H	OH	H

Compound **1** was obtained as colorless prisms and its HR-FABMS established a molecular formula of $C_{32}H_{50}O_4$. The IR spectrum showed characteristic absorption bands of hydroxy (3510 cm^{-1}), ester (1730 cm^{-1}) and ketone (1700 cm^{-1}) groups. The ^1H- and ^{13}C-NMR spectral data (Table 1) which were assigned from NMR techniques (DEPT, COSY, HMQC, HMBC and NOESY) suggested a lanostane-type triterpenoid with a C-3 ketone (δ_C = 218.2) and a side-chain possessing a *trans*-vinyl olefinic function [δ_H = 5.87 (1H, d, J = 15.6 Hz), δ_C = 142.6 and δ_H = 5.64 (1H, dd, J = 7.8, 15.6 Hz), δ_C = 121.1] with a methine group [δ_H = 5.31 (1H, dd, J = 3.5, 7.8 Hz), δ_C = 77.1] bearing an acetoxy moiety [δ_H = 2.06 (3H, s), δ_C = 24.4, 170.6] and a terminal tertiary alcohol (δ_C = 71.1]. The structure of **1** was established as (20*S*,22*S*,23*E*)-22-*O*-acetyl-25-hydroxylanosta-8,23(*E*)-dien-3-one from spectroscopic data and by comparison with those of related compounds [6], [7], [8], and was confirmed by X-ray diffraction (see Fig. **1**).

Bromination of **2** and **3** yielded two dibromo derivatives **4** and **5**, respectively. The ^1H- and ^{13}C-NMR spectra revealed the trisubstituted nature of each of the aromatic rings which were assigned by comparison with their parent spectral data and by 2D experiments (COSY, HMQC, HMBC and NOESY). The ES-MS which showed three sodiated molecular ions $[M + Na]^+$ at m/z = 575, 577 and 579 provided a molecular formula of $C_{22}H_{18}Br_2O_7$ for **4**, and those at m/z = 561, 563 and 565 corresponded to the molecular formula of $C_{21}H_{16}Br_2O_7$ for **5**. The NOESY spectra of both compounds exhibited correlations between 3″-OMe of **4** (and 3″-OH of **5**) to H-2 and H-6; H-5 to 4-OMe and H-6; 6″-OMe to H-2′ and H-6′; H-5′ to 4′-OMe and H-6′. These observations indicated that bromination occurred at C-3 and C-3′ in both **4** and **5**. Acetylation of **3** yielded acetyl 4,4′-dimethoxyvulpinate (**6**) and its IR spectrum showed the carbonyl absorptions of a vinyl acetate (1800 cm^{-1}), lactone (1780 cm^{-1}) and ester (1730 cm^{-1}) groups. The ^1H- and ^{13}C-NMR spectra were similar to those of **2**, except for the presence of an acetoxy moiety (δ_H = 2.29, δ_C = 20.6 and 165.1). The ES-MS at m/z = 447 $[M + Na]^+$ was consistent with structure **6**.

Compound **1** exhibited strong antiviral activity against *Herpes simplex* type 1 (HSV-1). Recently, antimycobacterial activity of

Table 1 ^1H- (400 MHz) and ^{13}C-(100 MHz) NMR spectral data (δ value, $CDCl_3$) of **1**

No.	*^1H*	*^{13}C*
1	1.62 m, 1.97 m	36.5 t[a]
2	2.41 ddd (3.6, 7.8, 15.7)[b], 2.59 ddd (7.1, 11.2, 15.7)	34.9 t
3	–	218.2 s
4	–	47.7 s
5	1.61 m	51.6 d
6	1.60 m	19.8 t
7	1.65 m, 1.95 m	27.5 t
8	–	135.5 s
9	–	133.7 s
10	–	37.3 s
11	2.03 m	21.5 t
12	2.08 m	26.8 t
13	–	45.1 s
14	–	49.9 s
15	1.70 m	31.3 t
16	1.25 m, 1.65 m	31.2 t
17	1.46 m	47.5 d
18	0.73 s	16.4 q
19	1.09 s	19.1 q
20	1.83 m	40.8 d
21	0.95 d (6.7)	13.7 t
22	5.31 dd (3.5, 7.8)	77.1 d
23	5.64 dd (7.8, 15.6)	121.1 d
24	5.87 d (15.6)	142.6 d
25	–	71.1 s
26	1.32 s	30.4[c] q
27	1.32 s	30.2[c] q
28	0.85 s	24.6 q
29	1.12 s	26.6 q
30	1.07 s	21.9 q
OCOMe	–	170.6 s
OCO*Me*	2.06 s	24.4 q

[a] Multiplicity deduced by DEPT and indicated by usual symbol.
[b] J values (in Hz) in parentheses.
[c] Assignments may be interchanged.

plant terpenoids has been reviewed [9], however **1** was inactive against *M. tuberculosis* H37Ra in our test. Compound **3** and its derivatives **5** and **6** exhibited antitubercular activity against *M. tuberculosis* H37Ra. In addition, compounds **5** and **6** also showed cytotoxicity towards lung cancer cell line NCI-H187 (Table 2).

Materials and Methods

The mushrooms, *S. citrinum*, were collected in Sakon Nakhon province, Thailand in August 1998 and identified by Assoc. Prof. K. Soytong. A voucher specimen (KMILT-SCL01) was deposited at the Department of Plant Pest Management, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. Fresh fruit bodies of *S. citrinum* (554 g) were ground and extracted with MeOH (3 × 700 mL) at room temperature for 2 days, then filtered. Filtrates were combined and the solvent was removed under vacuum to give a red brown residue (18.2 g, 3.28 %). The methanol

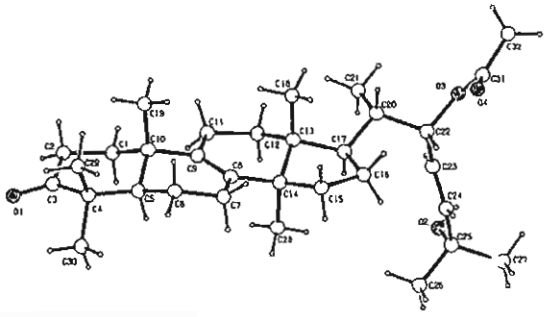

Fig. 1 Molecular structure of 1.

crude extract was subjected to CC over silica gel (360 g), gradient eluted with hexane-EtOAc (70:30, 1.5 L), hexane-EtOAc (60:40, 1 L), hexane-EtOAc (50:50, 3 L), hexane-EtOAc (40:60, 2 L), hexane-EtOAc (30:70, 2 L), hexane-EtOAc (10:90, 2 L), EtOAc-MeOH (80:20, 1 L), EtOAc-MeOH (50:50, 1 L) and MeOH (100%, 1 L). Fractions (150 mL) were collected and combined on the basis of TLC into 7 fractions (F_1-F_7). Fraction F_2 (hexane-EtOAc, 60:40, 0.5 L, 0.5 g) was recrystallized from EtOAc-hexane (1:1) to give **1** (195 mg). Fraction F_3 (hexane-EtOAc, 50:50, 3 L, 1.3 g) was recrystallized from EtOAc to yield yellow-green crystals of **2** (0.838 g). Fraction F_4 (hexane-EtOAc, 40:60, 2 L, 2.6 g) was recrystallized from EtOAc to yield an additional amount of **2** (0.692 g). The filtrate upon evaporation of the solvent and recrystallization of the residue from EtOAc yielded red-orange needles of **3** (0.763 g). An additional amount of **3** (1.135 g) was obtained from fraction F_5 (hexane-EtOAc, 10:90, 2 L, 2.2 g). Fraction 6 (EtOAc-MeOH, 80:20, 1 L, 2.7 g) and Fraction 7 (EtOAc-MeOH, 50:50, 1 L and MeOH 100%, 1 L, 4.1 g) contained an insoluble residue which unable to purify.

Compound **1**: m.p. 166 – 168 °C; $[\alpha]_D^{31}$: + 62.1° (c 0.15, $CHCl_3$); IR (KBr): ν_{max} = 3510, 2980, 2880, 1730, 1700, 1240 cm^{-1}; ^1H- and ^{13}C-NMR spectral data, see Table 1; HR-FAB-MS (positive ion mode): $C_{32}H_{50}O_4$ + H found: 449.3789, calcd.: 449.3787. Atomic coordinates, bond lengths, bond angles, and thermal parameters have been deposited at Cambridge Crystallographic Data Centre, England (CCDC No. 145134).

Compound **2**: m.p. 172 – 173 °C; UV (EtOH): λ_{max} (log ε) = 237 (4.27), 360 (4.41) nm; IR (KBr): ν_{max} = 3011, 1763, 1732, 1628, 1603, 1252, 1190, 1161, 845 cm^{-1}; The ^1H- and ^{13}C-NMR agree well with values reported in the literature [2], [3]; EI-MS: *m/z* = 396 $[M]^+$, 337, 309, 119, 89, 76; anal. found: C 66.64%, H 5.33%; calcd. for $C_{22}H_{20}O_7$: C 66.66%, H 5.09%.

Compound **4**: m.p. 208 – 209 °C; UV (EtOH): λ_{max} (log ε) = 206 (4.7), 230 (4.3), 357 (4.5) nm; IR (KBr): ν_{max} = 3110, 3005, 1780, 1735, 1630, 1600, 1500, 1265, 1235, 1170, 823 cm^{-1}; ^1H-NMR (400 MHz, $CDCl_3$): δ = 7.83 (1H, d, J = 2.3 Hz, H-2'), 7.77 (1H, d, J = 2.1 Hz, H-2), 7.68 (1H, dd, J = 8.8, 2.3 Hz, H-6'), 7.52 (1H, dd, J = 8.6, 2.1 Hz, H-6), 6.99 (1H, d, J = 8.7 Hz, H-5), 6.95 (1H, d, J = 8.8, Hz, H-5'), 3.95 (6H, s, 4,4'-*OMe*), 3.93 (3H, s, 6''-CO_2*Me*), 3.81 (3H, s, 3''-*OMe*); ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 167.8 (C-6''), 167.2 (C-1''), 162.8 (C-3''), 157.1 (C-4'), 156.8 (C-4), 141.2 (C-4''), 134.8 (C-2), 134.0 (C-2'), 130.5 (C-6'), 130.4 (C-6), 125.2 (C-1''), 122.2 (C-1), 115.0 (C-5''), 112.5 (C-3, C-3'), 112.2 (C-5'), 112.0 (C-5), 107.5 (C-2''), 61.7 (3''-*OMe*), 56.8 (4,4'-*OMe*), 53.3 (6''-CO_2*Me*).

Compound **5**: m.p. 208 – 209 °C; UV (EtOH): λ_{max} (log ε) = 205 (4.9), 304 (4.9), 383 (4.4) nm; IR (KBr): ν_{max} = 2555, 1770, 1678, 1620 1595, 1260, 1090, 830 cm^{-1}; ^1H-NMR (400 MHz, $CDCl_3$): δ = 8.42 (1H, d, J = 2.1 Hz, H-2), 8.16 (1H, dd, J = 8.7, 2.1 Hz, H-6), 7.47 (1H, d, J = 2.2 Hz, H-2'), 7.22 (1H, dd, J = 8.4, 2.2 Hz, H-6'), 6.98 (1H, d, J = 8.6 Hz, H-5), 6.95 (1H, d, J = 8.4 Hz, H-5'), 3.96 (6H, s, 4,4'-*OMe*), 3.93 (3H, s, 6''-CO_2*Me*), 13.52 (s, 3''-OH); ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 171.8 (C-6''), 166.0 (C-1''), 159.5 (C-3''), 156.5 (C-4), 156.2 (C-4''), 155.2 (C-4'), 135.1 (C-6'), 133.0 (C-2), 130.9 (C-6'), 128.6 (C-6), 125.7 (C-1'), 123.3 (C-1), 114.5 (C-5''), 112.2 (C-3), 112.0 (C-5), 111.8 (C-5'), 111.7 (C-3'), 104.3 (C-2'') 56.7 (4,4'-*OMe*), 54.9 (6''-CO_2*Me*).

Bioassay: The antiviral activity against *Herpes simplex* type 1 and cytotoxicity tests against human lung cancer cells NCI-H187 were performed employing the colorimetric method [10]. The re-

Table 2 Biological activities of compounds 1 – 6

Compounds	*Anti-HSV-1* IC_{50} *(μg/mL)*	*Anti-NCI-H187*[a] IC_{50} *(μg/mL)*	*Anti-*Mycobacterium *MIC (μg/mL)*
1	5.2	Inactive[b]	Inactive[c]
2	Inactive[b]	Inactive[b]	Inactive[c]
3	Inactive[b]	Inactive[b]	25
4	Inactive[b]	Inactive[b]	Inactive[c]
5	Inactive[b]	13.4	100
6	Inactive[b]	5.7	100
Acyclovir	2.0 – 5.0	–	–
Isoniazide	–	–	0.04 – 0.09
Kanamycin sulfate	–	–	2.0 – 5.0
Ellipticine	–	0.4 ± 0.1	–

[a] Human lung cancer cells.
[b] Inactive at 50 μg/mL
[c] Inactive at 200 μg/mL

ference substances were acyclovir and ellipticine for antiviral and anticancer cell lines, respectively (Table 2). The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA) [11]. In our system, the standard drugs isoniazide and kanamycin sulfate were the reference compounds (Table 2).

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Stereoselective synthesis and moulting activity of 2,3-*diepi*-20-hydroxyecdysone and 2,3-*diepi*-5α-20-hydroxyecdysone

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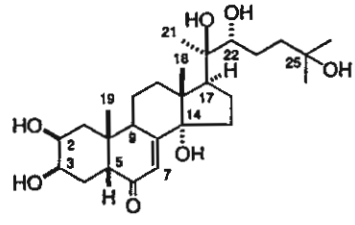
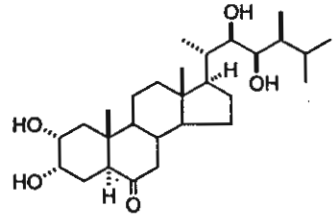
Abstract—The ecdysteroid analogues 2,3-*diepi*-20-hydroxyecdysone and 2,3-*diepi*-5α-20-hydroxyecdysone have been synthesized from the readily available ecdysteroid, 20-hydroxyecdysone, and moulting activity has been determined using the *Musca* bioassay. As expected, the 2,3-*diepi*-analogue was less active than the parent ecdysteroid, 20-hydroxyecdysone. However, the 2,3-*diepi*-5α-analogue, which was expected to be inactive in the assay, exhibited moulting activity though it was approximately 1.5-fold less active than its 5β-analogue. The activity of the 5α-analogue could possibly result from the ability of this compound to bind to the ecdysteroid receptor. Alternatively, a possible in vivo C-5 epimerization of the 2,3-*diepi*-5α-analogue to the corresponding 5β-analogue could account for its activity.

1. Introduction

Ecdysteroids are arthropod moulting hormones found in invertebrates and plant species and 20-hydroxyecdysone (**1**) is a representative of this class of compounds.[1–3] The physiological functions in invertebrates including insects are to control moulting and metamorphosis processes and are involved in the control of reproduction. Essential features contributing to high moulting activity include a *cis*-A/B ring junction, a 6-keto-7-ene system, a full sterol side chain and a free 14α-hydroxyl group.[4] The number, location and stereochemistry of the hydroxyl groups in the molecule are also responsible for the high activity of ecdysteroids. Many works indicated that the 3β-hydroxyl group is required for high activity of ecdysteroids and that the 2-hydroxyl group is not essential to such activity.[4–6] Previous works have shown that moulting activity of ecdysteroids decreased in going from the 3β-hydroxyl to the corresponding 3α-hydroxyl analogues.[4] All active ecdysteroids have a *cis*-fused A/B ring junction, whereas the 5α-epimers are inactive.[4,7] 5α-Ecdysteroid analogues, which have a *trans*-A/B ring junction, have an approximately planar ring structure while ecdysteroids (5β) are non-planar in the region of the A ring with C-2, C-3 and C-4 lying below the plane of the other rings.



1
12 2-Deoxy-5α-analogue of **1**



2

It was found that the brassinosteroid castasterone (**2**), the 7,8-dihydro analogue of ecdysteroid with the 2α,3α-dihydroxyl groups and 5α-orientation, inhibited ecdysteroid activity and the effects could be explained by competitive displacement of ecdysteroids, for example, compound **1**.[8] By comparing molecular models of **1** and **2** it was found that the 3β-hydroxyl group of **1** and the 3α-hydroxyl group of **2**

Keywords: Ecdysteroid; 2,3-*diepi*-20-Hydroxyecdysone; 2,3-*diepi*-5α-20-Hydroxyecdysone; Synthesis; Moulting activity.
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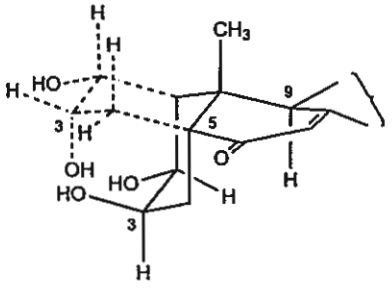
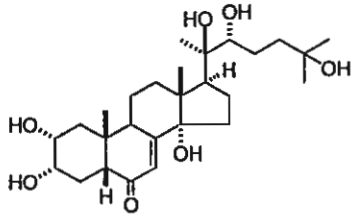
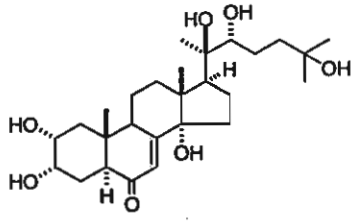


Figure 1. The A-ring geometry of the ecdysteroid 1 (solid line) and brassinosteroid 2 (broken line) and the near spatial coincidence of the 3β-hydroxyl group in 1 with the 3α-hydroxyl group in 2. Some hydrogens are omitted in the structure for clarity.

occupied the same space (Fig. 1) and this might lead to effective competition in binding the ecdysteroid receptor of 2. It was therefore of interest to study moulting activity of a 2,3-*diepi*-5α-ecdysteroid and, for comparison, the activity of a 2,3-*diepi*-ecdysteroid should also be evaluated. The present work deals with the stereoselective synthesis of 2,3-*diepi*-20-hydroxyecdysone (3) and 2,3-*diepi*-5α-20-hydroxyecdysone (4) from the readily available ecdysteroid 1 and evaluation of moulting activity of these two ecdysteroid analogues. Compound 4 possesses a structural framework in the A-ring region comparable to that of the brassinosteroid 2.



3



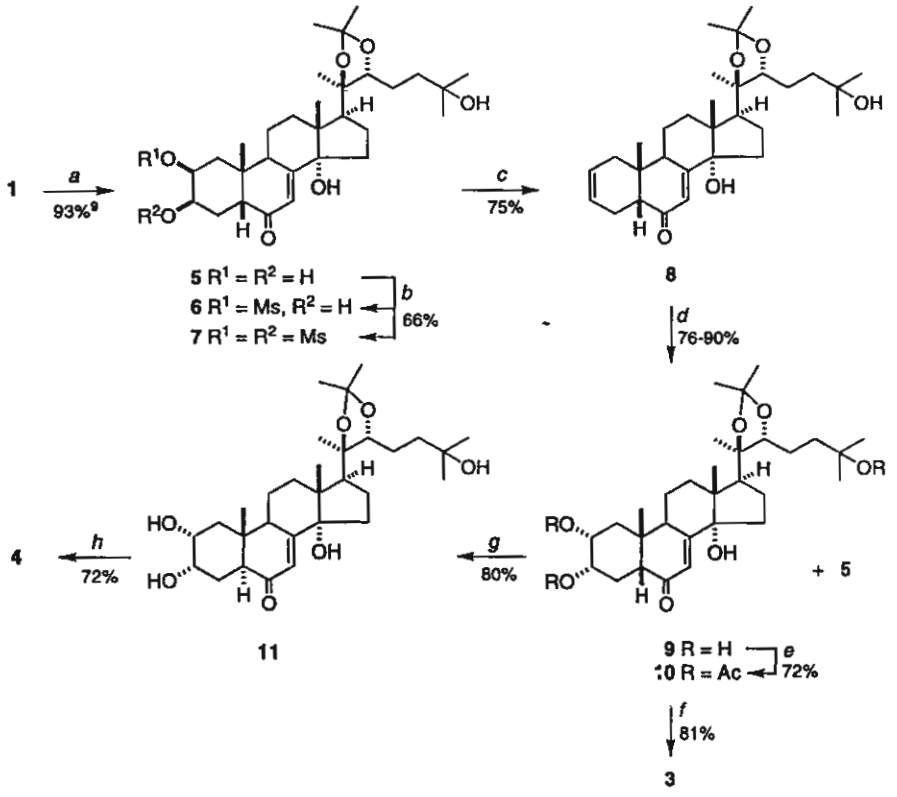
4

2. Results and discussion

The first ecdysteroid analogue we planned to synthesize was the 2,3-*diepi*-ecdysteroid 3. It was then expected that this compound would be transformed into the corresponding C-5 epimer, 2,3-*diepi*-5α-ecdysteroid 4, by base-catalyzed epimerization. Starting from 1, the 20,22-acetonide 5 was prepared by the literature procedure.[9] Mesylation of 5 with mesyl chloride in pyridine yielded the corresponding 2-mesylate 6[10] and 2,3-dimesylate 7 in 30 and 66% yields, respectively. The former could be recycled in the mesylation step. Prolonged reaction time gave only the dimesylate 7, but it was usually accompanied by a less polar side product. The presence of the mesylate groups at the 2- and 3-positions in 7 was evident from a 1.13 and 1.11 ppm downfield shifts of H-2 and H-3 as compared with that of the starting acetonide 5 and the presence of two mesylate methyl signals at δ 3.10 and 3.11 in the ^{1}H NMR spectrum of 7. Reaction of 7 with NaI and Zn in DMF at 80 °C afforded the olefin acetonide 8 in 75% yield. The ESMS exhibited a sodiated molecular ion $[M+Na]^+$ at *m/z* 509 and a pseudo-molecular ion $[M+H]^+$ at *m/z* 487, corresponding to a molecular formula of $C_{30}H_{46}O_5$. This was confirmed by positive-ion HRFABMS which exhibited a pseudo-molecular ion $[M+H]^+$at *m/z* 487.3428. The ^{1}H NMR spectrum of 8 indicated the presence of two olefinic (H-2 and H-3) signals at δ 5.52 and 5.69. The rest of the ^{1}H NMR spectroscopic data were consistent with structure (Scheme 1).

Dihydroxylation of the olefin acetonide 8 with OsO_4 in pyridine, followed by treatment with 5% aq. $NaHSO_3$ afforded two products in high yield. The more polar product (58% yield) was identified as 20-hydroxyecdysone 20,22-acetonide (5) by comparison of spectroscopic (IR, ^{1}H NMR and mass spectral) data with the reported value.[9] The less polar product (32% yield) was compound 9, the C-2 and C-3 epimer of 5. That the ecdysteroid 5 was the major product was expected, since dihydroxylation of 8 took place on the less-hindered β-face. The ^{1}H and ^{13}C NMR spectroscopic signals of 9 were assigned from 2D (COSY, HMQC, HMBC) and DEPT techniques and were found to be much different from those of 5. A striking difference was the unusual downfield H-9 signal of 9 (in C_5D_5N) at δ 4.70, whereas that of 5 appeared at δ 3.54. A close proximity of H-9 and the 2α-hydroxyl group resulted in a large downfield shift of the former signal. A marked upfield shift of the H-5 signal of 9 at δ 2.37, as compared with that of 5 at δ 2.99, was also noted. The strong steric interaction between the 2α-hydroxyl group and H-9 might lead to facile C-5 epimerization of 9. The structure of this minor dihydroxylation product could possibly, therefore, be 9 or its C-5 epimer of 9. To prove whether the stereochemistry at C-5 was in the β-orientation, a NOE experiment was performed. The key experiment was to irradiate the 19-Me signal to see if enhancement of the H-5 signal would occur.[11] The result, however, was not conclusive since the H-5 signal was obscured by other signals. Compound 9 was therefore subjected to acetylation to the corresponding 2,3,25-triacetate acetonide 10. Assignments of the NMR spectroscopic signals of 10 were achieved by 2D NMR techniques. Irradiation at the 19-Me frequency of 10 resulted in NOE enhancement of the H-5 signal at δ 2.20, whereas irradiation at the H-9 frequency caused enhancement of the 2α-acetoxyl signal at δ 1.98. To confirm that the position of the H-5 resonance was at δ 2.20, the H-3 resonance was irradiated and enhancement of this proton signal was also observed. The results indicated that H-5 of compound 10 was in the β-orientation. It was thus concluded that compound 9 was a 5β-ecdysteroid as shown. Acetonide deprotection of 9 with 70% AcOH in the presence of the phase transfer catalyst benzyltrimethylammonium chloride afforded 2,3-*diepi*-20-hydroxyecdysone (3) in 81% yield.

In order to increase the ratio of the product 9:5, asymmetric dihydroxylation[12] was investigated. The chiral ligands used were those of the dihydroquinidine (DHQD) series, that is,



Scheme 1. Synthesis of ecdysteroid analogues **3** and **4**. Reagents and conditions: (a) CH_3COCH_3, *p*-TsOH; (b) MsCl, pyridine; (c) NaI, Zn, DMF, 80 °C; (d) OsO_4, ligand, solvent (see text); (e) Ac_2O, pyridine; (f) 70% AcOH, $PhCH_2NMe_3^+Cl^-$; (g) 2% Na_2CO_3, MeOH; (h) 70% AcOH, $PhCH_2NMe_3^+Cl^-$.

dihydroquinidine 4-methyl-2-quinolyl ether (DHQD-MQE), dihydroquinidine 9-phenanthryl ether (DHQD-PE) and dihydroquinidine 1,4-phthalazinediyl diether $(DHQD)_2$-PHAL), and the dihydroquinine (DHQ) series, that is, DHQ-MQE, DHQ-PE and $(DHQ)_2$-PHAL (Table 1). The best **9**:**5** product ratio was 4:1, the chiral ligand of which was $(DHQD)_2$-PHAL. In this case the yield of **9** was raised to 68%. The overall yield of **3** from the starting ecdysteroid **1** was 25%, based on the utilization of the chiral ligand $(DHQD)_2$-PHAL in the dihydroxylation step.

To effect C-5 epimerization, compound **9** was treated with 2% Na_2CO_3 in MeOH and, as expected, epimerization occurred more readily than the 2β,3β-dihydroxyl analogue[7] (e.g., compound **5**) to give the corresponding 5α-analogue **11** in 80% yield. The *trans*-A/B ring fusion of **11** was evident from a large upfield shift (1.47 ppm) of H-9 in going from **9** to **11**. A downfield shift (0.65 ppm) of H-5 signal of **11** indicated the proximity of this proton and the 3α-hydroxyl group. The H-5 proton of **11** was further confirmed to be in the α-orientation by NOE experiments. Thus irradiation at the H-5 frequency did not cause enhancement of the 19-Me signal at δ 0.96, whereas irradiation at the H-9 frequency resulted in enhancement of the H-5 signal at δ 3.02. Compound **11** was subjected to deacetonation to give 2,3-*diepi*-5α-20-hydroxyecdysone (**4**) in 72% yield, the spectroscopic data of which were consistent with the structure. The overall yields of **4** from the starting ecdysteroid **1** was 18%, or 58% from compound **9**.

Table 1. Asymmetric dihydroxylation of compound **8**

Entry	Ligand	Ratio of products 5:9	Yield (%)
1	DHQD-MQE	5:6	77
2	DHQD-PE	7:3	83
3	$(DHQD)_2$-PHAL	1:4	85
4	DHQ-MQE	7:3	76
5	DHQ-PE	3:1	80
6	$(DHQ)_2$-PHAL	5:4	84

The ratio of the ligand:OsO_4: compound **8** was 3:3:1. *tert*-BuOH–THF–H_2O (7:4:1) was used as a solvent.

2.1. Biological activity

The *Musca* bioassay[13] has been used to evaluate moulting activity of ecdysteroid and their analogues in our study. As expected for a 3α-hydroxy ecdysteroid, compound **3** which is the 2α,3α-analogue of the parent ecdysteroid **1** was much less active; it was 30-fold less active than compound **1**. Surprisingly, compound **4**, the 5α-analogue of compound **3** and was expected to be inactive,[4] was active in the assay. It was 42-fold less active than compound **1**. The activity of **4**, though it was approximately 1.5-fold less active than that of **3**, deserved special attention. One possible explanation for the activity of **4** was that it could bind to the ecdysteroid receptor by analogy to that which occurred in castasterone (**2**). Unlike **2**, compound **4** possesses a 6-keto-7-ene system, the essential structural requirement for moulting activity. The α-nature of the 2- and 3-hydroxyl groups resulted in effective binding to the receptor and this could possibly

compensate the *trans*-A/B ring junction of **4**. An alternative explanation was that compound **4** could undergo in vivo C-5 epimerization to compound **3**. The unfavorable steric interaction of the 2α-hydroxyl group and H-9 in **3** could, in part, be compensated for by the absence of 1,3-diaxial interaction between 19-Me and the 2β- and 4β-H (see Fig. 1). One supported example was the activity of compound **12**, a 2-deoxy-5α-ecdysteroid analogue, which exhibited low moulting activity in the *Musca* assay.[14] Whether the first or second hypothesis was more likely could not be judged from the existing data.

3. Experimental

3.1. General experimental procedures

Melting points were determined on an Electrothermal melting point apparatus and were uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 spectrometer. FAB and ES mass spectra were measured on a Finnigan MAT 90 and a Bruker Esquire-LC instruments. Column chromatography and TLC were carried out using Merck silica gel 60 (<0.063 mm) and precoated silica gel 60 F_{254} plates, respectively. Spots on TLC were visualized under UV light and by spraying with anisaldehyde–H_2SO_4 reagent followed by heating.

3.1.1. 20-Hydroxyecdysone 20,22-acetonide 2,3-dimesylate (7). Compound 5[9] (1.4 g, 2.69 mmol) was dissolved in pyridine (4 mL) and the mixture stirred at 0–5 °C for 10 min, then mesyl chloride (1.5 mL, 19.30 mmol) was added. The reaction mixture was left to stir at 0–5 °C for 1 h and at ambient temperature for another 1 h. Water was added and the mixture extracted with $CHCl_3$ (4×25 mL). The combined chloroform extract was washed with water, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was subjected to column chromatography (silica gel 50 g) using $CHCl_3$–MeOH, with gradually increasing concentration of MeOH, to give 20-hydroxyecdysone 20,22-acetonide 2,3-dimesylate (**7**) (1.2 g, 66%) eluted by $CHCl_3$–MeOH (98.5:1.5) and 20-hydroxyecdysone 20,22-acetonide 2-mesylate (**6**) (490 mg, 30%), eluted by $CHCl_3$–MeOH (96:4).

Compound **6**. Spectroscopic (^1H NMR and mass spectral) data were identical to those reported in literature.[10]

Compound **7**. Amorphous; ν_{max} 3436, 2971, 1660, 1449, 1354, 1176, 1106, 1024, 909 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.77 (s, 3H, 18-Me), 1.03 (br s, 3H, 19-Me), 1.14 (s, 3H, 21-Me), 1.22 (s, 3H, 26-Me), 1.23 (s, 3H, 27-Me), 1.31, 1.39 (each s, 2×3H, acetonide Me), 2.22 (dd, *J*=9.1, 7.9 Hz, 1H, H-17), 2.98 (m, 1H, H-9), 3.10, 3.11 (each s, 2×3H, 2-OMs, 3-OMs), 3.63 (dd, *J*=9.4, 2.4 Hz, 1H, H-22), 4.94 (m, 1H, H-2), 5.12 (br s, 1H, H-3), 5.87 (d, *J*=2.1 Hz, 1H, H-7); ESMS *m/z* (% rel. intensity) 699 $[M+Na]^+$(100)]; HRFABMS (positive ion mode) *m/z* 677.3045 $[M+H]^+$ (calcd for $C_{32}H_{52}O_{11}S_2$–H, 677.3029).

3.1.2. 2,3-Didehydro-2,3-dideoxy-20-hydroxyecdysone 20,22-acetonide (8). Compound **7** (960 mg, 1.42 mmol) was dissolved in DMF (5 mL) and NaI (820 mg, 5.47 mmol) and zinc dust (196 mg, 3 mmol) was added. The reaction mixture was left to stir at 80 °C for 3 days. Water was added and the mixture was extracted with $CHCl_3$. The combined $CHCl_3$ layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to dryness. The product was purified by column chromatography (silica gel 30 g) using $CHCl_3$–MeOH with gradually increasing concentration of MeOH to give compound **8** (520 mg, 75%), eluted by $CHCl_3$–MeOH (99:1).

Compound **8**. Colorless needles (from $CHCl_3$–MeOH), mp 117–119 °C; ν_{max} 3426, 2971, 1659, 1443, 1376, 1213, 1200 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.75 (s, 3H, 18-Me), 1.02 (s, 3H, 19-Me), 1.12 (s, 3H, 21-Me), 1.20 (s, 3H, 26-Me), 1.21 (s, 3H, 27-Me), 1.29, 1.38 (each s, 2×3H, acetonide Me), 2.22 (t, *J*=ca 9 Hz, 1H, H-17), 2.85 (br m, 1H, H-9), 3.63 (br d, *J*=7.8 Hz, 1H, H-22), 5.50 and 5.67 (each br d, *J*=10 Hz, 2×1H, H-2 and H-3), 5.76 (d, *J*=1.3 Hz, 1H, H-7); ESMS (positive ion mode) *m/z* (% rel. intensity) 995 $[2M+Na]^+$ (25), 509 $[M+Na]^+$ (100), 487 $[M+H]^+$ (17); HRFABMS (positive ion mode) *m/z* 487.3428 $[M+H]^+$ (calcd for $C_{30}H_{46}O_5$+H, 487.3423).

3.1.3. Reaction of compound 8 with osmium tetroxide. Synthesis of 2,3-*diepi*-20-hydroxyecdysone 20,22-acetonide (9) and 20-hydroxyecdysone 20,22-acetonide (5). To a solution of compound **8** (134 mg, 0.275 mmol) in pyridine (0.8 mL) was added OsO_4 in pyridine (0.55 mL, prepared by dissolving 500 mg of OsO_4 in 3 mL of pyridine and the amount used was equivalent to 92 mg or 0.36 mmol of OsO_4). The mixture was stirred for 5 min and 5% $NaHSO_3$ (2 mL) was added. Stirring was continued for 15 min and the mixture was extracted with EtOAc (3×15 mL). The combined organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude mixture (150 mg) was subjected to column chromatography (silica gel 25 g), using $CHCl_3$–MeOH as eluting solvent, with increasing amount of MeOH. Fractions eluted by $CHCl_3$–MeOH (95:5) afforded 46 mg (32%) of 2,3-*diepi*-20-hydroxyecdysone 20,22-acetonide (**9**). The second fraction (83 mg, 58%) eluted by $CHCl_3$–MeOH (94:6), was identified as 20-hydroxyecdysone 20,22-acetonide (**5**).

Compound **5**. Colorless needles (from acetone–hexane), mp 221–223 °C (lit.[9] 222–224 °C); ν_{max} 3423, 2974, 1649, 1454, 1377, 1216, 1170, 1103, 1057, 1001, 877 cm^{-1}; ^1H NMR (400 MHz, C_5D_5N) δ 1.00, 1.03 (each s, 2×3H, 18-Me, 19-Me), 1.32 (s, 3H, acetonide Me),[a] 1.34 (s, 3H, 26-Me), 1.35 (s, 3H, 27-Me), 1.44 (s, 3H, acetonide Me), 1.53 (s, 3H, 21-Me),[a] 2.75 (t, *J*=8.6 Hz, 1H, H-17), 2.99 (dd, *J*=13.1, 3.5 Hz, 1H, H-5), 3.54 (m, 1H, H-9), 3.93 (dd, *J*=9.6, 2.4 Hz, 1H, H-22), 4.16 (m, 1H, H-2), 4.22 (br s, 1H, H-3), 6.24 (d, *J*=2.1 Hz, 1H, H-7), ('a' stands for the assignments may be reversed for signals with the same superscript); ^1H NMR (400 MHz, $CDCl_3$) δ 0.76 (s, 3H, 18-Me), 0.93 (s, 3H, 19-Me), 1.12 (s, 3H, 21-Me), 1.18 (s, 3H, 26-Me),[b] 1.26 (s, 3H, 27-Me),[b] 1.29,[b] 1.38 (each s, 2×3H, acetonide Me), 2.38 (dd, *J*=12.9, 4.4 Hz, 1H, H-5), 2.95 (m, 1H, H-9), 3.59 (m, 1H, H-22), 3.81 (m, 1H, H-2), 4.01 (br s, 1H, H-3), 5.81 (d, *J*=1.8 Hz, 1H, H-7), ('b' stands for the assignments may be reversed for signals with the same superscript); ESMS (positive ion mode) *m/z* (% rel. intensity) 543 $[M+Na]^+$ (100); HRFABMS (negative ion

mode) *m/z* 519.3322 $[M-H]^-$. (calcd for $C_{30}H_{48}O_7-H$, 519.3321).

Compound **9**. Amorphous; ν_{max} 3418, 2970, 1654, 1458, 1384, 1258, 1200, 1173, 1075, 1002, 927, 867 cm^{-1}; ^1H NMR (400 MHz, C_5D_5N) δ 0.99 (br s, 3H, 19-Me), 1.03 (s, 3H, 18-Me), 1.30 (s, 3H, 21-Me), 1.37 (s, 2×3H, 26-Me, 27-Me), 1.45, 1.53 (each s, 2×3H, acetonide Me), 2.37 (obscured signal, 1H, H-5), 2.77 (t, J=8.3 Hz, 1H, H-17), 3.92 (obscured signal, 1H, H-3), 3.94 (dd, J=9.3, 2.8 Hz, 1H, H-22), 4.33 (br s, $W_{1/2}$=12 Hz, 1H, H-2), 4.70 (br, 1H, H-9), 6.22 (d, J=2.3 Hz, 1H, H-7); ^{13}C NMR (100 MHz, C_5D_5N) δ 17.3 (C-18), 21.3 (C-16), 22.1 (C-11), 22.4 (C-21), 24.2 (C-19), 24.4 (C-23), 27.2 (acetonide Me), 29.4 (acetonide Me), 29.7 (C-4), 29.8 (C-26), 30.1 (C-27), 31.6 (C-12), 31.8 (C-15), 36.2 (C-9), 36.6 (C-10), 39.9 (C-1), 42.1 (C-24), 47.9 (C-13), 49.9 (C-17), 57.7 (C-5), 69.2 (C-25), 70.4 (C-2), 71.5 (C-3), 82.5 (C-22), 84.0 (C-14), 85.1 (C-20), 106.9 (acetonide C), 121.1 (C-7), 167.8 (C-8), 202.1 (C-6); HRFABMS (negative ion mode) *m/z* 519.3324 $[M-H]^-$. (calcd for $C_{30}H_{48}O_7-H$, 519.3321).

3.2. Asymmetric dihydroxylation of 8 with OsO_4 and chiral ligands

General procedure. To a solution of 0.03 mmol of a chiral ligand in *tert*-BuOH–THF–H_2O (7:4:1, 0.6 mL) was added a THF solution of OsO_4 (14 μL, 0.03 mmol. The solution was prepared by dissolving 500 mg of OsO_4 in 9 mL of THF.) and the mixture stirred for 3 min. A solution of the olefin acetonide **8** (5 mg, 0.01 mmol) in *tert*-BuOH–THF–H_2O (7:4:1, 0.5 mL) was then added and stirring continued for 5 min. The ratio of the ligand, OsO_4 and olefin acetonide was 3:3:1. A 5% solution of $NaHSO_3$ (10 mL) was added and stirring continued for another 10 min. The mixture was extracted with EtOAc (4×20 mL); the combined organic phase was evaporated and the residue was chromatographed to separate compounds **5** and **9** from the ligand. Since the two products could easily be separated from each other by column chromatography, the **5**:**9** ratio for each ligand was determined from the isolated products **5** and **9**. The results are shown in Table 1.

3.2.1. Acetylation of compound 9. A mixture of compound **9** (9 mg, 0.017 mmol), Ac_2O (0.1 mL, 1.05 mmol) and pyridine (0.7 mL) was stirred for 6 h. The reaction mixture was worked up in the usual manner and the product purified by column chromatography to give 2,3-*diepi*-20-hydroxyecdysone 2,3,25-triacetate (**10**) (8 mg, 72%).

Compound **10**. Amorphous; ν_{max} 3482, 2977, 1744, 1666, 1458, 1370, 1246, 1168, 1137, 1106, 928, 874 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.76 (s, 3H, 18-Me), 0.94 (br s, 3H, 19-Me), 1.12 (s, 3H, 21-Me), 1.29, 1.38 (each s, 2×3H, acetonide Me), 1.43 (s, 3H, 26-Me), 1.45 (s, 3H, 27-Me), 1.97 (s, 2×3H, 3-OAc, 25-OAc), 1.98 (s, 3H, 2-OAc), 2.20 (partially obscured signal, 1H, H-5), 3.59 (dd, J=ca 9, 2.5 Hz, 1H, H-22), 3.75 (br, $W_{1/2}$=21 Hz, 1H, H-9), 4.88 (br, $W_{1/2}$=20 Hz, 1H, H-3), 5.26 (br s, $W_{1/2}$=13 Hz 1H, H-2), 5.86 (d, J=1.9 Hz, 1H, H-7); ^{13}C NMR (100 MHz, C_5D_5N) δ 17.1 (C-18), 20.9 (acetate Me), 21.1 (2×C, acetate Me), 21.9 (C-21), 23.2 (C-16), 23.6 (C-19), 25.7 (C-26), 26.1 (C-27), 26.8 (acetonide Me), 28.9 (acetonide Me), 30.9, 31.9 (C-12, C-15), 35.0 (C-9), 36.1 (C-10), 36.7 (C-1), 38.5 (C-24), 47.2 (C-13), 49.0 (C-17), 55.8 (C-5), 69.3, 71.0 (C-2, C-3), 81.4 (C-22), 82.0 (C-25), 84.0 (C-20), 84.9 (C-14), 106.8 (acetonide C), 120.9 (C-7), 165.8 (C-8), 170.0 (2×C, acetate CO), 170.5 (acetate CO), 200.6 (C-6); HRFABMS (positive ion mode) *m/z* 647.3787 $[M+H]^+$. (calcd for $C_{36}H_{54}O_{10}+H$, 647.3787).

3.2.2. Acetonide deprotection of compound 9. Compound **9** (36 mg, 0.069 mmol) was dissolved in 70% AcOH (0.7 mL, excess) and benzyltrimethylammonium chloride (25 mg, 0.135 mmol) was added. The reaction mixture was left to stir for 4 h; water was then added and the mixture extracted with *n*-BuOH (3×15 mL). The combined organic layer was washed with water; the solvent was removed by co-evaporation with water under reduced pressure. The crude product was purified by column chromatography using $CHCl_3$–MeOH as eluting solvent to afford 2,3-*diepi*-20-hydroxyecdysone (**3**) (27 mg, 81%).

Compound **3**. Colorless needles (from MeOH–EtOAc), mp 204–206 °C; ν_{max} 3420, 2965, 1654, 1383, 1065, 929 cm^{-1}; ^1H NMR (400 MHz, C_5D_5N) δ 1.00 (s, 3H, 19-Me), 1.22 (s, 3H, 18-Me), 1.38 (s, 2×3H, 26-Me, 27-Me), 1.54 (s, 3H, 21-Me), 2.40 (obscured signal, 1H, H-5), 3.00 (t, J=8.9 Hz, 1H, H-17), 3.87 (br d, J=8.7 Hz, 1H, H-22), 3.90 (obscured signal, 1H, H-3), 4.34 (br s, $W_{1/2}$=8.5 Hz, 1H, H-2), 4.76 (br m, 1H, H-9), 6.22 (d, J=2.6 Hz, 1H, H-7); ^{13}C NMR (100 MHz, C_5D_5N) δ 18.0 (C-18), 21.4 (C-16), 21.5 (C-11), 21.6 (C-21), 24.2 (C-19), 27.5 (C-23), 29.8 (C-4), 29.9 (C-26), 30.3 (C-27), 31.8 (C-12), 32.1 (C-15), 36.3 (C-9), 36.7 (C-10), 39.9 (C-1), 42.7 (C-24), 48.2 (C-13), 50.1 (C-17), 57.8 (C-5), 69.6 (C-25), 70.5 (C-2), 71.6 (C-3), 76.9 (C-20), 77.5 (C-22), 84.1 (C-14), 121.0 (C-7), 168.4 (C-8), 202.2 (C-6); HRFABMS (negative ion mode) *m/z* 479.3001 $[M-H]^-$. (calcd for $C_{27}H_{44}O_7-H$, 479.3008).

3.2.3. Epimerization of compound 9. A mixture of compound **9** (35 mg, 0.067 mmol) in MeOH (0.8 mL) and 2% Na_2CO_3 (0.2 mL, 0.038 mmol) was stirred at ambient temperature for 5 h and water was then added. The solution was extracted with *n*-BuOH (3×10 mL); the combined butanol layer was washed with water and the solvent removed by co-evaporation with water. The product was purified by column chromatography to afford 2,3-*diepi*-5α-20-hydroxyecdysone 20,22-acetonide (**11**) (28 mg, 80%).

Compound **11**. Amorphous; ν_{max} 3422, 2971, 2942, 1664, 1458, 1375, 1219, 1175, 1105, 1054, 1001, 905, 868 cm^{-1}; ^1H NMR (400 MHz, C_5D_5N) δ 0.95 (s, 3H, 19-Me), 0.99 (s, 3H, 18-Me), 1.30 (s, 3H, 21-Me), 1.35 (s, 2×3H, 26-Me, 27-Me), 1.43, 1.52 (each s, 2×3H, acetonide Me), 2.74 (t, J=ca 8 Hz, 1H, H-17), 3.02 (br d, J=11.1 Hz, 1H, H-5), 3.23 (m, 1H, H-9), 3.93 (br d, J=8.5 Hz, 1H, H-22), 4.02 (m, $W_{1/2}$=21 Hz, 1H, H-2), 4.42 (br s, $W_{1/2}$=8 Hz, 1H, H-3), 6.16 (br s, 1H, H-7); ^{13}C NMR (100 MHz, C_5D_5N) δ 13.6 (C-19), 17.3 (C-18), 20.8 (C-16), 22.0 (C-11), 22.4 (C-21), 24.4 (C-23), 27.2 (acetonide Me), 28.2 (C-4), 29.5 (acetonide Me), 29.9 (C-26), 30.1 (C-27), 31.5 (C-12), 31.6 (C-15), 40.3 (C-10), 41.2 (C-1), 42.2 (C-24), 46.7 (C-9), 47.6 (C-13), 48.7 (C-5), 49.9 (C-17), 68.3 (C-2), 69.1 (C-3), 69.3 (C-25), 82.5 (C-22), 83.9 (C-14), 85.1 (C-20), 106.9 (acetonide C), 123.3 (C-7), 164.4 (C-8), 201.7 (C-6);

HRFABMS (negative ion mode) *m/z* 519.3313 $[M-H]^-$. (calcd for $C_{30}H_{48}O_7-H$, 519.3322).

3.2.4. Acetonide deprotection of compound 11. Compound **11** (15 mg, 0.029 mmol) was subjected to acetonide deprotection in the same manner as described for the preparation of **3** from **9**. The product was purified by column chromatography to afford 2,3-*diepi*-5α-20-hydroxyecdysone (**4**) (10 mg, 72%).

Compound **4**. Amorphous; ν_{max} 3415, 2925, 1660, 1384, 1062 cm^{-1}; ^1H NMR (400 MHz, C_5D_5N) δ 0.84 (s, 3H, 19-Me), 1.21 (s, 3H, 18-Me), 1.38 (s, 2×3H, 26-Me, 27-Me), 1.58 (s, 3H, 21-Me), 2.99 (t, J=9.2 Hz, 1H, H-17), 3.03 (dd, J=12.4, 3.6 Hz, 1H, H-5), 3.28 (m, 1H, H-9), 3.89 (br d, J=8.9 Hz, 1H, H-22), 4.04 (m, $W_{1/2}$=20 Hz, 1H, H-2), 4.44 (br s, $W_{1/2}$=9 Hz, 1H, H-3), 6.18 (d, J=2.4 Hz, 1H, H-7); ^{13}C NMR (100 MHz, C_5D_5N) δ 13.6 (C-19), 17.9 (C-18), 20.9 (C-16),[a] 21.4 (C-11),[a] 21.7 (C-21), 27.4 (C-23),[b] 28.3 (C-4),[b] 29.9 (C-26), 30.2 (C-27), 31.8 (C-12, C-15), 40.3 (C-10), 41.3 (C-1), 42.7 (C-24), 46.7 (C-9), 47.9 (C-13), 48.7 (C-5), 50.1 (C-17), 68.4 (C-2), 69.1 (C-3), 69.6 (C-25), 76.9 (C-20), 79.8 (C-22), 84.0 (C-14), 123.3 (C-7), 164.9 (C-8), 201.8 (C-6), ('a and b' stand for assignments may be reversed for signals with the same superscript); HRFABMS (negative ion mode) *m/z* 479.3018 $[M-H]^-$. (calcd for $C_{27}H_{44}O_7-H$, 479.3008).

3.5. Moulting bioassay

Compounds **3** and **4** were subjected to the *Musca* bioassay, using *Musca domestica* larvae.[13] The purity of the ecdysteroid and their analogues was checked by reversed-phase HPLC. The bioassay results were scored[15] and EC_{50}, the molar concentration of each steroid required to effect puparium formation of 50% effectiveness, of each compound was determined by plotting concentrations against % effectiveness of puparium formation.[16] The EC_{50} values of compounds **3** and **4** were 5.0×10^{-4} and 7.0×10^{-4} M, respectively, whereas that of the reference compound **1** was 1.65×10^{-5} M.

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(+)-Bornyl Piperate, a New Monoterpene Ester from *Piper* aff. *pedicellatum* Roots

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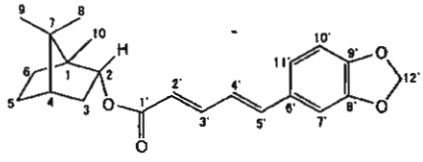
A new monoterpene ester, (+)-bornyl piperate was isolated from the underground roots of *Piper* aff. *pedicellatum* and its structure was elucidated on the basis of spectroscopic evidence and confirmed by X-ray analysis. The compound crystallizes in the triclinic space group *P*1 with a=7.3232(4) Å, b=11.4705(7) Å, c=23.2520(14) Å, V=1943.6(2) Å^3. This compound showed an antituberculosis activity against *Mycobacterium tuberculosis* (H_{37}Ra strain) with the minimum inhibitor concentration (MIC) of 25 μg/ml.

Key words *Piper* aff. *pedicellatum*; Piperaceae; antituberculosis; *Mycobacterium tuberculosis*

Piper aff. *pedicellatum* C. DC. (Piperaceae) is distributed mainly in Northern Thailand. Its roots and stems are used as a carminative in Thai folk medicine. Although there are many reports on the phytochemistry of the genus *Piper*,[1] no previous record on the chemical constituents of this plant species has been found in the literature. As part of our ongoing project on bioactive compounds from Thai medicinal plants for the treatment of tropical diseases, we have investigated this plant species. We now describe the isolation and structure elucidation of a new monoterpene ester, (+)-bornyl piperate (**1**) together with eight known compounds (**2**—**9**) from the hexane and the methanol extracts of the underground roots of *P.* aff. *pedicellatum*.

The powdered roots of *P.* aff. *pedicellatum* were extracted successively with *n*-hexane and MeOH in a Soxhlet apparatus. The hexane extract on chromatography over silica gel gave one new compound (**1**) and six known compounds (**2**—**7**), while chromatography of the methanol extract afforded two more known compounds (**8**—**9**). The known compounds were identified as a mixture of β-sitosterol (**2**)[2] and stigmasterol (**3**),[2] pellitorine (**4**),[3] guineensine (**5**),[4] pipernonaline (**6**),[5] piperine (**7**)[6] and a mixture of β-sitosteryl-3-*O*-β-glucopyranoside (**8**)[7] and stigmasteryl-3-*O*-β-glucopyranoside (**9**)[8] by comparison of their physical and spectroscopic data with those reported in the literature.



1

Chart 1

Compound **1** was obtained as colorless needles and its molecular formula was determined to be $C_{22}H_{26}O_4$ by HR-FAB-MS (*m/z* 355.1905, $[M+H]^+$). Its IR spectrum displayed an absorption band at 1710 cm^{-1}, indicating the presence of a conjugated ester group. The ^{1}H- and ^{13}C-NMR spectra exhibited the presence of the 2*E*,4*E*-piperoyl moiety.[6] The remaining ^{1}H- and ^{13}C-NMR signals of **1** were very similar to those reported for the bornyl group of bornyl *p*-coumarate isolated from *P. ribesioides*.[9] In the HMBC spectrum, a long range correlation was observed between the piperoyl carbonyl carbon C-1′ (δ 167.5) and the H-2 proton (δ 4.96) of the bornyl moiety. The stereochemistry at C-2 was determined by means of a NOE experiment. Upon irradiation of H-2, a NOE enhancement was observed for H-3β, 8-CH_3 and 10-CH_3, showing that the piperoyl group is located at the *endo* side of the bornyl moiety. Moreover, the X-ray structure (Fig. 1) confirmed the relative stereochemistry of **1**. It should be noted that the X-ray crystal structure of (+)-bornyl *p*-coumarate isolated from *P. caninum*[10] has recently been established. Therefore, the structure of **1** was established as 2-*endo*-bornyl piperate.

Compound **1** exhibited an antituberculosis activity against *Mycobacterium tuberculosis* (H_{37}Ra strain)[11] with the MIC of 25 μg/ml.

Experimental

General Procedures Melting points were determined on an Electrothermal apparatus and are uncorrected. UV spectra were measured with a Perkin Elmer Lamda 20 spectrophotometer. IR spectra were obtained with a Perkin

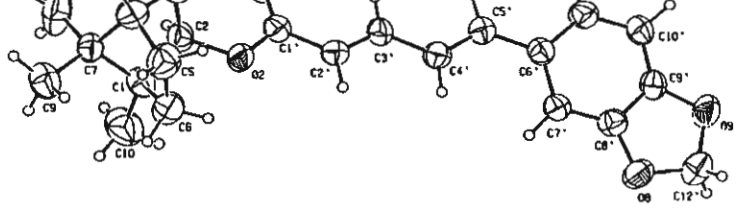


Fig. 1. ORTEP Perspective Drawing of **1**

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...er Spectrum 2000 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AVANCE 400 NMR spectrometer, operating at 400 and 100 MHz, respectively. Mass spectra were recorded with a Finnigan MAT 90 instrument. Column chromatography and TLC were carried out using Merck silica gel 60 (>230 mesh) and precoated silica gel 60 F_{254} plates, respectively. Spots on TLC were visualized under UV light and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating.

Plant Material The underground roots of *P.* aff. *pedicellatum* were collected from Pasang district, Lamphun province, Thailand in December, 1999. A voucher specimen (No. BKF 93307) has been deposited at the herbarium of the Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok.

Extraction and Isolation The air-dried, powdered roots of *P.* aff. *pedicellatum* (314.5 g) were extracted successively with *n*-hexane and MeOH using a Soxhlet apparatus. The hexane and MeOH extracts were evaporated to dryness *in vacuo*. The hexane extract (7.44 g) was subjected to a silica gel column (150 g) using a gradient solvent system of hexane, hexane–EtOAc and EtOAc (5% increment of the polar solvent for each 250 ml of mobile phase) to give 20 main fractions. Fr. 5 (193 mg) was rechromatographed on a silica gel column (40 g) using EtOAc–hexane (1.5 : 98.5) (1.5 l) to give 5 subfractions. Fr. 5.3 yielded **1** (12 mg). Fr. 9 (209 mg) was fractionated into 4 subfractions on a siliga gel column (8 g) using EtOAc–hexane (8 : 92) (300 ml) as eluent. Subsequent filtration of fr. 9.3 afforded a mixture of **2**[2] and **3**[3] (63 mg). Repetitive CC (silica gel, 25 g) of fr. 12 (293 mg) using EtOAc–hexane (80 : 20) (1.5 l) as eluent gave 4 subfractions. Fr. 12.3 yielded **4**[4] (44 mg). Fr. 14 (211 mg) was further purified on a silica gel column (10 g) eluting with EtOAc–hexane (80 : 20) (300 ml) to give 7 subfractions. Compound **5**[4] (13 mg) was isolated from fr. 14.4. Fr. 16 (110 mg) was rechromatographed on a silica gel column (7 g) using EtOAc–hexane (30 : 70) (300 ml) as eluent to give 6 subfractions. Fr. 16.4 afforded **6**[5] (50 mg) while filtration of fr. 18 (123 mg) furnished **7**[6] (71 mg). The MeOH extract (7.37 g) was subjected to CC (silica gel, 150 g) eluting with a gradient of $CHCl_3$, $CHCl_3$–MeOH and MeOH in increasing proportions of the polar solvent (each 200 ml) to give 9 main fractions. Fr. 7 (540 mg) was further purified on a silica gel column (30 g) eluting with MeOH–$CHCl_3$ (2 : 98) (2 l) to give 15 subfractions. Fr. 7.12 (136 mg) was filtered to give a mixture of **8**[7] and **9**[8] (56 mg).

Bornyl Piperate (**1**): Colorless needles; mp 93—95 °C (hexane); $[\alpha]_D^{27}$ +7.80° (c=0.1, $CHCl_3$). UV λ_{max} (MeOH) nm: 261, 316, 353. IR (KBr) ν_{max} cm^{-1}: 3024, 2951, 1710, 1624, 1502, 1489, 1447, 1375, 1325, 1253, 1196, 1175, 1139, 1037, 995, 931, 880, 848, 799, 754, 724. EI-MS *m/z* (rel. int.): 354 (M^+, 30), 218 (38), 202 (14), 201 (100), 173 (18), 143 (11), 137 (27), 115 (13), 81 (26), 69 (11). HR-FAB-MS *m/z*: 355.1905 (Calcd for $C_{22}H_{26}O_4$ $[M+H]^+$: 355.1909). ^{1}H-NMR ($CDCl_3$) δ: 0.84 (3H, s, 10-CH_3), 0.87 (3H, s, 9-CH_3), 0.91 (3H, s, 8-CH_3), 1.01 (1H, dd, J=13.1, 4.3 Hz, H-3α), 1.24 (1H, m, H-5α), 1.33 (1H, m, H-6β), 1.68 (1H, brt, J=4.3 Hz, H-4), 1.74 (1H, m, H-5β), 2.00 (1H, m, H-6α), 2.38 (1H, m, H-3β), 4.96 (1H, br ddd, J=13.1, 2.6, 2.6 Hz, H-2), 5.96 (1H, d, J=15.3 Hz, H-2'), 5.97 (2H, s, H-12'), 6.69 (1H, dd, J=15.7, 10.8 Hz, H-4'), 6.77 (1H, d, J=8.2 Hz, H-10'), 6.80 (1H, d, J=15.7 Hz, H-5'), 6.90 (1H, dd, J=8.2, 1.5 Hz, H-11'), 6.98 (1H, d, J=1.5 Hz, H-7'), 7.38 (1H, dd, J=15.3, 10.8 Hz, H-3'). ^{13}C-NMR ($CDCl_3$) δ: 13.5 (C-10), 18.9 (C-8), 19.7 (C-9), 27.2 (C-6), 28.1 (C-5), 36.9 (C-3), 45.1 (C-4), 47.8 (C-1), 48.9 (C-7), 79.7 (C-2), 101.4 (C-12'), 105.9 (C-7'), 108.5 (C-10'), 121.1 (C-2'), 122.8 (C-11'), 124.6 (C-4'), 130.7 (C-6'), 139.9 (C-5'), 144.3 (C-3'), 148.3 (C-9')*, 148.5 (C-8')*; 167.5 (C-1'). (Values with an asterisk are interchangeable.)

Crystal Data of (+)-Bornyl Piperate (**1**): $C_{22}H_{26}O_4$, MW 354.45, Triclinic, $P1$, a=7.3232(4) Å, b=11.4705(7) Å, c=23.2520(14) Å, V=1943.6(2) Å^3. A total of 7259 unique reflections (6,528 observed, $|F_o|>4\sigma|F_o|$) were measured at room temperature from a 0.30×0.15×0.05 mm^3 colorless crystal using graphite monochromated MoKα radiation (λ=0.71073 Å) on a Bruker-Nonius kappa CCD diffractometer. With Z=4, the asymmetric unit contains four molecules of bornyl piperate with the calculated density of 1.211 g cm^{-3}. The crystal structure was solved by direct methods using SIR-97, and then all atoms except hydrogen atoms were refined anisotropically on F^2 using SHELXL-97 to give a final R-factor of 0.0561 (R_w=0.1470) with a data-to-parameter ratio of 7.74 : 1. Atomic coordinates, bond lengths, bond angles, and thermal parameters have been deposited with the Cambridge Crystallographic Data Center, 12 Union Road, Cambridge, CB2 1EZ, ENGLAND (CCDC 214540).

Bioassay Procedure The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* $H_{37}Ra$ strain using the Microplate Alamar Blue Assay (MABA).[11] The MIC values of the standard drugs isoniazid and kanamycin sulfate are 0.050 and 2.5 μg/ml, respectively.

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Chemical constituents and bioactivity of *Piper sarmentosum*

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Abstract

Eight amides, pellitorine (**1**), guineensine (**2**), brachystamide B (**3**), sarmentine (**4**), brachyamide B (**5**), 1-piperettyl pyrrolidine (**6**), 3′,4′,5′-trimethoxycinnamoyl pyrrolidine (**7**) and sarmentosine (**8**), two lignans, (+)-asarinin (**9**) and sesamin (**10**), and four other compounds, 1-(3,4-methylenedioxyphenyl)-1E-tetradecene (**11**), methyl piperate (**12**) and a mixture of β-sitosterol (**13**) and stigmasterol (**14**), were isolated from the fruits of *Piper sarmentosum* (Piperaceae). This is the first reported isolation of compounds **2**, **3**, **5**, **6**, **7**, **9**, **10** and **12** from this plant species. Their structures were established from spectral data. These compounds were evaluated in antituberculosis and antiplasmodial tests. The results showed that compounds **4** and **6** exhibited both activities while compounds **1**, **2**, **5**, **8** and **11** showed only antituberculosis activity. This is the first report of the antituberculosis and antiplasmodial activities for these compounds.

Keywords: *Piper sarmentosum*; Piperaceae; Amides; Lignans; Antituberculosis activity; Antiplasmodial activity

1. Introduction

The genus *Piper*, widely distributed in the tropical and subtropical region of the world, is often used as food flavouring agents, traditional medicines (Burkill, 1966) and pest control agents (Nair and Burke, 1990). Phytochemical investigations of *Piper* species have led to the isolation of several classes of physiologically active compounds such as alkaloids, amides, pyrones, dihydrochalcones, flavonoids, phenylpropanoids, lignans and neolignans (Parmar et al., 1997).

Piper sarmentosum Roxb. (Piperaceae), locally known as "Cha-plu", is a glabrous, creeping terrestrial herb about 20 cm tall. The plant and fruits are used in Thailand as an expectorant (Pongboonrod, 1976). The ethanolic extract of the leaves has been reported to reduce the blood sugar in alloxan diabetic rabbits (Pongmarutai, 1980). In the Malay and Indonesian Archipalago, the leaves and roots of this plant are used for the treatment of toothace, fungoid dermatitis on the feet, coughing asthma and pleurisy (Perry, 1981). The water extract of the whole plant showed a hypoglycemic effect in rats (Peungvicha et al., 1998) while the methanolic extract of the leaves was found to possess a marked neuromuscular blocking activity in rat phrenic nerve-hemidiaphragm preparation (Ridtitid et al., 1998). In addition, the chloroform and methanol extracts of the leaves showed considerable antiplasmodial activity against *Plasmodium falciparum* and *Plasmodium berghei* parasites (Najib Nik et al., 1999). Previous phytochemical studies on this plant have resulted in the isolation of a number of amides and phenylpropanoids (Masuda et al., 1991; Likhitwitayawuid et al., 1987; Stohr et al., 1999). We now describe the isolation of chemical constituents from the fruits of *Piper sarmentosum* and results of the antituberculosis and antiplasmodial tests on some isolates.

2. Material and methods

2.1. General experimental procedures

UV spectra were measured with a Perkin-Elmer Lamda 20 spectrophotometer; IR spectra were obtained with a Perkin-Elmer Spectrum 2000 spectrophotometer; ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 NMR spectrometer, operating at 400 and 100 MHz,

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spectively; EIMS were determined on a Finnigan MAT INCOS 50 spectrometer; ESMS were determined on a Bruker ESQUIRE-LC spectrometer.

2.2. Plant material

Fruits of *Piper sarmentosum* were collected from Kanchanaburee province, Thailand. A voucher specimen (No. 0039 (RU)) is deposited at the Faculty of Science, Ramkhamhaeng University.

2.3. Extraction and separation

The pulverized, dried fruits of *Piper sarmentosum* (2.39 kg) were extracted successively with hexane and MeOH in a Soxhlet apparatus. After removal of solvent in vacuo, the hexane extract (70.7 g) was subjected to quick CC (silica gel) eluting with hexane, hexane–$CHCl_3$, $CHCl_3$, $CHCl_3$–MeOH and MeOH (in order of increasing polarity) to give 6 main fractions (H1–H6). Fraction H2 was rechromotographed on silica gel column using a gradient solvent system of hexane, hexane–EtOAc, EtOAc, EtOAc–MeOH and MeOH to obtain 13 subfractions (H2.1–H2.13). Fraction H2.2 was further purified by silica gel CC using hexane as eluent to give **11** (284 mg) whereas fraction H2.7 yielded a mixture of **13** and **14** (13 mg). Fraction H2.9 was rechromatographed on silica gel column using EtOAc–hexane (15:75) as eluent to afford **1** (122 mg). Fraction H3 was separated by silica gel CC using a hexane–EtOAc step gradient to obtain 24 subfractions (H3.1–H3.24). Filtration of fractions H3.4 and H3.17 yielded **9** (5 mg) and **2** (91 mg), respectively. Fractions H4 and H5 were separately fractionated into 12 subfractions each (H4.1–H4.12 and H5.1–H5.12, respectively) on silica gel columns using a gradient solvent system similar to that of fraction H2. Purification of fraction H4.4 by chromatography on silica gel and elution with EtOAc–hexane (15:85) afforded **3** (6 mg) whereas fraction H4.6 yielded **4** (1.28 g). From fraction H5.11, **5** (14 mg) was isolated by silica gel CC using EtOAc–hexane (25:75) as eluent.

The MeOH extract (99.11 g) was fractionated into 23 main fractions (M1–M23) by the method similar to that of the crude hexane extract. Fraction M1 was further purified by silica gel CC eluting with hexane to give **11** (152 mg). Fraction M3 was separated by chromatotron using hexane–$CHCl_3$ (95:5) as eluent to obtain 5 subfractions (M3.1–M3.5). Fraction M3.4 yielded **12** (10 mg). Repetitive CC (silica gel) of fraction M3.5 using a gradient solvent system of hexane–EtOAc afforded **10** (2 mg). Fraction M4 was fractionated into 6 subfractions (M4.1–M4.6) on a silica gel column using a gradient of hexane–EtOAc (100:0–15:85) as eluent. Fraction M4.1 was further purified by silica gel CC eluting with a gradient of hexane–EtOAc (100:0–96.5:3.5) to give **1** (158 mg). Fraction M8 was separated by chromatotron using hexane–EtOAc (90:10) as eluent to give 8 subfractions (M8.1–M8.8). Fractions M8.5 and M8.7 yielded **6** (224 mg) and **7** (88 mg). Compound **8** (318 mg) was isolated from fraction M9 by silica gel CC using a gradient of $CHCl_3$–MeOH (100:0–10:90) as eluent.

2.4. Bioassays

2.4.1. Antiplasmodial activity

Antiplasmodial activity was evaluated against the parasite *Plasmodium falciparum* (K1, multidrug resistant strain) which was cultured continuously according to the method of Trager and Jensen (1976). Quantitative assessment of antiplasmodial activity in vitro was determined by means the microculture radioisotope technique based upon the method described by Desjardins et al. (1979). An IC_{50} value of 1 ng/ml was observed for the standard compound, artemisinin, in the same test system.

2.4.2. Antituberculosis activity

The antituberculosis activity was assessed against *Mycobacterium tuberculosis* H37Ra strain using the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). The MIC values of the standard drugs isoniazid and kanamycin sulfate are 0.050 and 2.5 μg/ml, respectively.

3. Results and discussion

Eight amides, two lignans and four other compounds were isolated from the hexane and methanol extracts of *Piper sarmentosum* fruits and identified by spectroscopic methods: pellitorine (**1**) (Likhitwitayawuid et al., 1987), guineensine (**2**) (Okogun and Ekong, 1974; Koul et al., 1988), brachystamide B (**3**) (Banerji and Das, 1989), sarmentine (**4**) (Likhitwitayawuid et al., 1987), brachyamide B (**5**) (Koul et al., 1988), 1-piperettyl pyrrolidine (**6**) (Singh et al., 1974), 3′,4′,5′-trimethoxycinnamoyl pyrrolidine (**7**) (Achenbach et al., 1986), sarmentosine (**8**) (Likhitwitayawuid et al., 1987), (+)-asarinin (**9**) (Pelter et al., 1976), sesamin (**10**) (Pelter et al., 1976; Anjaneyulu et al., 1977), 1-(3,4-methylenedioxyphenyl)-1E-tetradecene (**11**) (Likhitwitayawuid et al., 1987), methyl piperate (**12**) (Kijjoa et al., 1989) and a mixture of β-sitosterol (**13**) (Pouchert and Behnke, 1993) and stigmasterol (**14**) (Pouchert and Behnke, 1993). The structures of compounds **1–14** are presented in Fig. 1.

This is the first report of the isolation of compounds **2**, **3**, **5**, **6**, **7**, **9**, **10** and **12** from *Piper sarmentosum*. These compounds were earlier isolated from other *Piper* species (Parmar et al., 1997). Nine isolated compounds were assessed for antituberculosis and antiplasmodial activities, the results of which are presented in Table 1. It revealed that most of the tested amides exhibited the antituberculosis activity whereas only two amides, **4** and **6**, also showed antiplasmodial activity. The results suggest that the presence of a *N*-pyrrolidinyl 2E,4E-dienamide moiety plays an

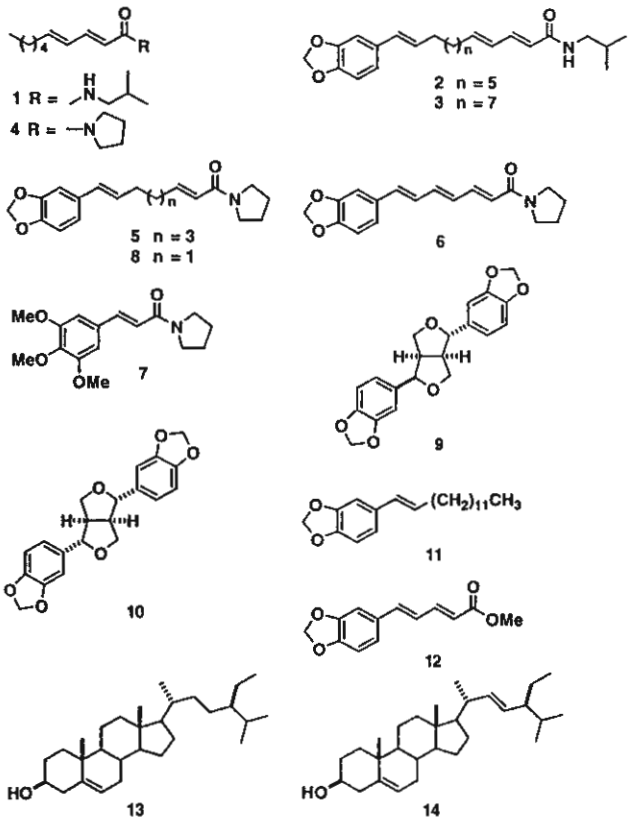

Fig. 1. Structures of isolated compounds from *Piper sarmentosum*.

important role in the antiplasmodial activity. In the case of the antituberculosis activity, the presence of either an unsaturated amide function with a 3,4-methylenedioxystyryl terminal group, or a 2E,4E-dienamide function with a terminal alkyl chain is essential. The exception is for compound **11** containing a 3,4-methylenedioxystyryl moiety and a terminal alkyl chain instead of the unsaturated amide moiety.

Table 1
Antituberculosis and antiplasmodial activities of some isolated compounds from *Piper sarmentosum*

Compounds	Antituberculosis activity MIC (μg/ml)	Antiplasmodial activity IC_{50} (μg/ml)
1	25	Inactive[a]
2	50	Inactive[a]
4	100	18.9
5	50	Inactive[a]
6	50	6.5
7	Inactive[b]	Inactive[a]
8	200	Inactive[a]
11	25	Inactive[a]
12	Inactive[b]	Inactive[a]

[a] Inactive at ≥20 μg/ml.
[b] Inactive at >200 μg/ml.

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Antimycobacterial Activity and Cytotoxicity of Flavonoids from the Flowers of *Chromolaena odorata*

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From the flowers of *Chromolaena odorata* (*Eupatorium odoratum*) four flavanones, isosakuranetin (5,7-dihydroxy-4'-methoxyflavanone) (**1**), persicogenin (5,3'-dihydroxy-7,4'-dimethoxyflavanone) (**2**), 5,6,7,4'-tetramethoxyflavanone (**3**) and 4'-hydroxy-5,6,7-trimethoxyflavanone (**4**), two chalcones, 2'-hydroxy-4,4',5',6'-tetramethoxychalcone (**5**) and 4,2'-dihydroxy-4',5',6'-trimethoxychalcone (**6**), and two flavones, acacetin (5,7-dihydroxy-4'-methoxyflavone) (**7**) and luteolin (5,7,3',4'-tetrahydroxyflavone) (**8**) were isolated and identified. Compound **1** exhibited moderate antimycobacterial activity against *Mycobacterium tuberculosis* with the MIC value of 174.8 μM, whereas compounds **4**, **7**, and **8** exhibited weak activity with the MIC values of 606.0, 704.2 and 699.3 μM respectively. Compound **7** showed moderate cytotoxicity against human small cell lung cancer (NCI-H187) cells with the MIC value of 24.6 μM, whereas compound **8** exhibited moderate toxicity against NCI-H187 cells and week toxicity against human breast cancer (BC) cells with the MIC values of 19.2 and 38.4 μM respectively.

Key words: *Chromolaena odorata*, Asteraceae, Flavonoids, Antimycobacterial activity, Cytotoxicity, Struture-activity relationship

INTRODUCTION

Chromolaena odorata (L.) R. M. King & H. Robinson (synonym: *Eupatorium odoratum* L.) is a perennial scandent or semi-woody shrub belonging to the Asteraceae family. This plant species is native to central and south America and it is now distributed throughout Africa and tropical Asia (Muniappan and Marutani, 1991). In traditional medicine, a decoction of the leaf is used as a cough remedy and as an ingredient with lemon grass and guava leaves for the treatment of malaria. The juice pressed out of the crushed leaves is applied to cuts to stop bleeding. Other medicinal uses include antidiarrheal, astringent, antispasmodic, antihypertensive, antiinflammatory and diuretic (Iwu, 1993). In Thailand, leave juice is used as a haemostatic on wounds and antiinflammatory. A decoction of flowers is used as tonic, antipyretic and heart tonic (Bunyapraphatsara and Chokechaijaroenporn, 2000). Previous investigations of the leaves and stems of *C. odorata* revealed the presence of essential oils (Inya-Agha *et al.*, 1987; Lamaty *et al.*, 1992; Bamba *et al.*, 1993; Chowdhury, 2002), steroids (Ahmad *et al.*, 1967), triterpenes (Tarapatra *et al.*, 1974; Tarapatra *et al.*, 1977), flavonoids (Bose *et al.*, 1973; Tarapatra *et al.*, 1974; Bose *et al.*, 1974; Tarapatra *et al.*, 1977; Arene *et al.*, 1978; Barua *et al.*, 1978; Metwally *et al.*, 1981; Hai *et al.*, 1991; Triratana *et al.*, 1991; Hai *et al.*, 1995; Wollenweber *et al.*, 1995; Wollenweber and Roitman, 1996). Flowers of this plant species have been subjected to investigation for essential oils (Baruah and Leclercq, 1993), fats (Baruah and Pathak, 1993) and alkaloids (Biller *et al.*, 1994). We now report on the flavonoid constituents of the flowers of *C. odorata* and some biological evaluations of the isolated flavonoids.

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MATERIALS AND METHODS

General experimental procedures

Melting points were determined on an Electrothermal apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer FT-IR Spectrum BX spectrophotometer. ^{1}H-NMR spectra were recorded on a Bruker AVANCE 400 spectrometer. The residual nondeuterated solvent, $CHCl_3$, at 7.24 ppm was used as a reference. Electron impact (EI) mass spectra were measured with a Thermo Finnigan Polaris Q spectrometer. Unless indicated otherwise, Merck silica gel 60 (finer than 0.063 mm) was used for column chromatography. TLC was conducted on plates precoated with Merck silica gel 60 F_{254}. Spots on TLC were visualized under UV light and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating.

Plant material

The flowers of *C. odorata* were collected from Mai-khao, Talang district, Phuket province, in February, 1999. A voucher specimen of this plant, No. 0033 (RU), is deposited at the Faculty of Science, Ramkhamhaeng University.

Extraction and isolation

The dried flowers of *C. odorata* (1.3 kg) was pulverized and extracted successively with hot *n*-hexane, $CHCl_3$ and MeOH (3 L×4 for each extract) to give the hexane (27.54 g), $CHCl_3$ (28.79 g) and MeOH (46.89 g) extracts, respectively. The $CHCl_3$ extract (25.00 g) was chromatographed over silica gel (0.063-0.200 mm, 300 g), eluting with $CHCl_3$, $CHCl_3$-EtOAc, EtOAc, EtOAc-MeOH, with gradually increasing quantity of the more polar solvent. The eluates were examined by TLC and 18 groups of eluting fractions (C1-C18) were obtained. Fraction C6 (601 mg) was chromatographed (silica gel, 25 g) using *n*-hexane-EtOAc as eluent, with increasing percentage of the more polar solvent, to give 13 subfractions. The 10th subfraction was chromatographed, eluted by *n*-hexane-EtOAc with increasing quantity of the more polar solvent, to yield compound 2 (1 mg). Fraction C7 (762 mg) was chromatographed (silica gel, 30 g) using *n*-hexane-$CHCl_3$, $CHCl_3$ and $CHCl_3$-MeOH as eluting solvents, to give 9 subfractions. The 6th subfraction was rechromatographed, with *n*-hexane-EtOAc and EtOAc as eluting solvents, to yield compound 5 (2 mg). The 7th subfraction was subjected to column chromatography in similar manner to that of the previous subfraction to give compound 3 (2 mg). The 8th subfraction was similarly rechromatographed to afford compound 1 (6 mg) and compound 6 (2 mg).

The MeOH extract (25.00 g) was chromatographed (silica gel, 0.063-0.200 mm, 300 g), eluting with EtOAc-MeOH under gradient condition to give 18 groups of eluting fractions (M1-M18). Fraction M7 (167 mg) was chromatographed (silica gel, 15 g), eluting with EtOAc and EtOAc-MeOH, to yield 8 subfractions. The 6th subfraction was rechromatographed twice to give compound **7** (2 mg). Fraction M9 (312 mg) was similarly subjected to two repeated column chromatographies to yield compound **4** (2.5 mg). Fraction M12 (1.03 g) was rechromatographed twice, using EtOAc and EtOAc-MeOH as eluents, to give compound **8** (8 mg).

Isosakuranetin (1)

White needles, mp: 178-179°C; IR ν_{max} cm^{-1}: 3158, 2956, 1636, 1598, 1518, 1496, 1302, 1253, 1164, 833; EIMS *m/z* (% rel. intensity): 286 $[M]^+$ (62), 152 (95), 134 (100), 124 (10); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.77 (1H, dd, J = 17.1, 3.0 Hz, H-3a), 3.09 (1H, dd, J = 17.1, 13.0 Hz, H-3b), 3.81 (3H, s, 4'-OMe), 5.33 (1H, dd, J = 13.0, 3.0 Hz, H-2), 5.84 (1H, br s, 7-OH), 5.95 (1H, d, J = 2.2 Hz, H-6), 5.97 (1H, d, J = 2.2 Hz, H-8), 6.93 (2×1H, d, J = 8.7 Hz, H-3', H-5'), 7.35 (2×1H, d, J = 8.7 Hz, H-2', H-6').

Persicogenin (2)

IR ν_{max} cm^{-1}: 3450, 2916, 2848, 1636, 1515, 1458, 1273, 1205, 1156; EIMS *m/z* (% rel. intensity): 316 $[M]^+$ (100), 286 (15), 167 (72), 150 (30), 149 (11), 137 (65); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.77 (1H, dd, J = 17.1, 3.0 Hz, H-3a), 3.06 (1H, dd, J = 17.1, 12.9 Hz, H-3b), 3.78 (3H, s, 7-OMe), 3.90 (3H, s, 4'-OMe), 5.31 (1H, dd, J = 12.9, 3.0 Hz, H-2), 5.66 (1H, br s, 3'-OH), 6.03 (1H, d, J = 2.2 Hz, H-6), 6.05 (1H, d, J = 2.2 Hz, H-8), 6.86 (1H, d, J = 8.3 Hz, H-5'), 6.91 (1H, dd, J = 8.3, 2.0 Hz, H-6'), 7.03 (1H, d, J = 2.0 Hz, H-2'), 11.95 (1H, s, 5-OH).

5,6,7,4'-Tetramethoxyflavanone (3)

IR ν_{max} cm^{-1}: 2934, 2847, 1677, 1600, 1516, 1484, 1457, 1261, 1200, 1106, 1020; EIMS *m/z* (% rel. intensity): 344 $[M]^+$ (72), 210 (100), 195 (78), 167 (33), 134 (2); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.74 (1H, dd, J = 16.6, 2.2 Hz, H-3a), 3.01 (1H, dd, J = 16.6, 13.3 Hz, H-3b), 3.80, 3.81, 3.84, 3.92 (each 3H, each s, 5-OMe, 6-OMe, 7-OMe, 4'-OMe), 5.33 (1H, dd, J = 13.3, 2.2 Hz, H-2), 6.32 (1H, s, H-8), 6.93 (2×1H, d, J = 8.5 Hz, H-3', H-5'), 7.36 (2×1H, d, J = 8.5 Hz, H-2', H-6').

4'-Hydroxy-5,6,7-trimethoxyflavanone (4)

EIMS *m/z* (% rel. intensity): 330 $[M]^+$ (98), 210 (87), 195 (100), 167 (65), 120 (10); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.74 (1H, dd, J = 16.6, 2.8 Hz, H-3a), 2.99 (1H, dd, J = 16.6, 13.3 Hz, H-3b), 3.80, 3.85, 3.92 (each 3H, each s, 5-OMe, 6-OMe, 7-OMe), 5.31 (1H, dd, J = 13.3, 2.8 Hz, H-2), 6.32 (1H, s, H-8), 6.86 (2×1H, d, J = 8.5 Hz, H-3', H-5'), 7.31 (2×1H, d, J = 8.5 Hz, H-2', H-6').

2'-Hydroxy-4,4',5',6'-tetramethoxychalcone (5)
EIMS *m/z* (% rel. intensity): 344 $[M]^+$ (35), 210 (100), 195 (71), 167 (27); ^{1}H-NMR (400 MHz, $CDCl_3$) 3.81, 3.84, 3.88, 3.90 (each 3H, each s, 4-OMe, 4'-OMe, 5'-OMe, 6'-OMe), 6.27 (1H, s, H-3'), 6.92 (2×1H, d, J = 8.8 Hz, H-3, H-5), 7.58 (2×1H, d, J = 8.8 Hz, H-2, H-6), 7.82, 7.83 (each 1H, each br s, H-α, H-β), 13.74 (1H, s, 2'-OH).

4,2'-Dihydroxy-4',5',6'-trimethoxychalcone (6)
EIMS *m/z* (% rel. intensity): 330 $[M]^+$ (100), 210 (91), 195 (80), 167 (34); ^{1}H-NMR (400 MHz, $CDCl_3$) 3.81, 3.88, 3.91 (each 3H, each s, 4'-OMe, 5'-OMe, 6'-OMe,), 5.14 (1H, br s, 4-OH), 6.27 (1H, s, H-3'), 6.85 (2×1H, d, J = 8.6 Hz, H-3, H-5), 7.54 (2×1H, d, J = 8.6 Hz, H-2, H-6), 7.80, 7.81 (each 1H, each br s, H-α, H-β), 13.72 (1H, s, 2'-OH).

Acacetin (7)
IR ν_{max} cm^{-1}: 3450, 2927, 1654, 1560, 1508, 1241, 1190, 1165; EIMS *m/z* (% rel. intensity): 284 $[M]^+$ (100), 283 (7), 256 (2), 152 (5), 132 (20), 124 (4), 117 (4), 89 (4); ^{1}H-NMR (400 MHz, $CDCl_3$+CD_3OD, 15:1) δ: 3.84 (3H, s, 4'-OMe), 6.22 (1H, d, J = 1.9 Hz, H-6), 6.38 (1H, d, J = 1.9 Hz, H-8), 6.51 (1H, br s, H-3), 6.96 (2×1H, d, J = 8.8 Hz, H-3', H-5'), 7.79 (2×1H, d, J = 8.8 Hz, H-2', H-6').

Luteolin (8)
mp: 330-332°C; IR ν_{max} cm^{-1}: 3423, 2920, 1654, 1611, 1500, 1367, 1267, 1164, 1032, 839; EIMS *m/z* (% rel. intensity): 286 $[M]^+$ (100), 258 (26), 153 (14), 134 (7); ^{1}H-NMR (400 MHz, $CDCl_3$+CD_3OD, 10:1) δ: 6.21 (1H, d, J = 2.0 Hz, H-6), 6.37 (1H, d, J = 2.0 Hz, H-8), 6.45 (1H, s, H-3), 6.88 (1H, d, J = 8.3 Hz, H-5'), 7.31 (1H, dd, J = 8.3, 2.1 Hz, H-6'), 7.33 (1H, d, J = 2.1 Hz, H-2').

Antimycobacterial assay

The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). Antimycobacterial evaluations of the flavonoids **1** and **3-8** are shown in Table I.

Cytotoxicity assays

The cytotoxicity assays against human epidermoid carcinoma of the mouth (KB), human breast cancer (BC) and human small cell lung cancer (NCI-H187) cells were performed employing colorimetric method (Skehan *et al.*, 1990). The cytotoxicity evaluations of the flavonoids **1** and **3-8** are shown in Table II.

RESULTS AND DISCUSSION

Identification of flavonoids

Four flavanones, **1**, **2**, **3**, and **4**, were isolated from the flowers of *C. odorata*. The ^{1}H-NMR and EI mass spectral data of compound **1** was consistent with 5,7-dihydroxy-4'-methoxyflavanone (isosakuranetin) isolated previously from the leaves of *C. odorata* (Bose *et al.*, 1973). NOE experiment confirmed that the methoxyl group was located at the 4'-position.

Compound **2** was a minor flavanone isolated from this plant species. The ^{1}H-NMR features of **2** were similar to those of compound **1**, except for those of the B-ring which showed a 1,3,4-trisubstituted pattern. The methoxyl group was proven to be at the 4'-position, as judged from the NOE enhancement of H-5' resonance upon irradiation of the methoxyl resonance at δ 3.90. From the spectroscopic (^{1}H-NMR and EI mass spectra) evidence, compound **2** was concluded to be 5,3'-dihydroxy-7,4'-dimethoxyflavanone (persicogenin) isolated previously from *E. sternburgianum* (Gonzalez *et al.*, 1982).

Compound **3** exhibited similar ^{1}H-NMR spectral pattern to that of compound **1**, except for that of the A-ring which

Table I. Antimycobacterial activity of flavonoids

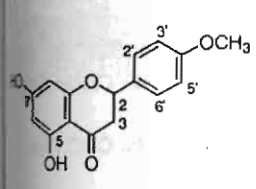
Compound	MIC (μM)
1	174.8
3	inactive[a]
4	606.0
5	inactive[a]
6	inactive[a]
7	704.2
8	699.3
Kanamycin sulfate[b]	4.29
Isoniazid[b]	0.44

[a]Inactive at > 200 μg/mL (> 581.4 μM for compounds **3** and **5**, and > 606.0 μM for compound **6**)
[b]Positive control

Table II. Cytotoxicity of flavonoids

Compound	IC_{50} (μM)		
	KB	BC	NCI-H187
1	inactive[a]	inactive[a]	inactive[a]
3	inactive[a]	inactive[a]	inactive[a]
4	inactive[a]	inactive[a]	inactive[a]
5	inactive[a]	inactive[a]	inactive[a]
6	inactive[a]	inactive[a]	inactive[a]
7	inactive[a]	inactive[a]	24.6
8	inactive[a]	38.4	19.2
Ellipticine[b]	5.40	5.93	1.58

[a]Inactive at > 20 μg/mL (> 69.9 μM for compounds **1** and **8**, > 58.1 μM for compounds **3** and **5**, > 60.6 μM for compounds **4** and **6**, and > 70.4 μM for compound **7**)
[b]Positive control



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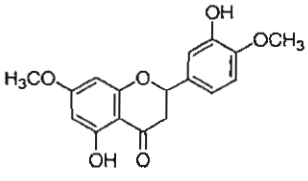

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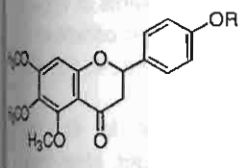

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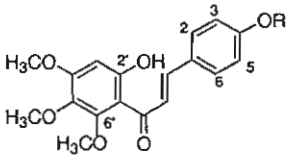
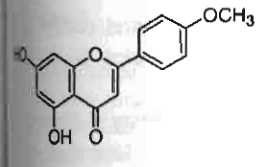
4 R = H



5 R = CH_3
6 R = H



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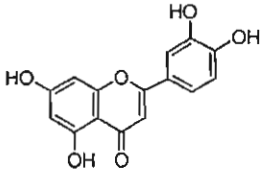

8

showed only one proton as a singlet signal of H-8 at δ 6.32. The ^{1}H-NMR spectral features of compounds **3** and **4** are very similar, except for the presence of an additional methoxyl group in the former. The presence of the fragment ion peak at *m/z* 134 in the EI mass spectrum of **3**, as compared to *m/z* 120 in that of **4** indicated that compound **4** was the 4'-demethoxyl analog of compound **3**. Compound **3** and **4**, therefore, were 5,6,7,4'-tetramethoxyflavanone and 4'-hydroxy-5,6,7-trimethoxyflavanone respectively. These compounds were isolated previously from the aerial parts of *C. odorata* (Barua *et al.*, 1978).

The ^{1}H-NMR spectroscopic data of compounds **5** and **6** showed characteristics of chalcones, the substitution pattern of the A-ring of which was the same as that of compounds **3** and **4**. The EI mass spectral fragmentation confirmed the identity of compounds **5** and **6** with 2'-hydroxy-4,4',5',6'-tetramethoxychalcone and 4,2'-dihydroxy-4',5,6-trimethoxychalcone isolated respectively from the leaves (Bose *et al.*, 1973) and aerial parts (Barua *et al.*, 1978) of *C. odorata*.

The third group of flavonoids are the flavones **7** and **8**. The ^{1}H-NMR and mass spectral data of compound **7** were consistent with those of acacetin isolated previously from the leaves of this plant (Bose *et al.*, 1974). From the spectroscopic (IR, ^{1}H-NMR and EI mass spectra) data, compound **8** was concluded to be luteolin (Pollock and Stevens, 1965; Mabry *et al.*, 1970), a common flavone isolated from this plant species for the first time.

Biological activities of the isolated flavonoids

Antimycobacterial activity

Compounds **1** and **3-8** were subjected to antimycobacterial evaluations (Table I). The quantity of compound **2** was not sufficient for biological evaluations. The flavanone **1** exhibited moderate antimycobacterial activity against *Mycobacterium tuberculosis*, with the MIC value of 174.8 μM, whereas the flavanone **4**, and the flavones **7** and **8** exhibited weak activity with the MIC values of 606.0, 704.2 and 699.3 μM respectively. Since the flavanones **1** and **4**, and the flavones **7** and **8** were active in the test, it was likely that a double bond at the C-ring was not essential for antimycobacterial activity. Both the chalcones **5** and **6** were inactive in the test, it followed that one of the structural requirements for a flavonoid to exhibit antimycobacterial activity was the presence of ring C, either saturated or unsaturated. The inactivity of the flavanone **3** and the positive bioassay results of compounds **1** and **4** needed special attention. Comparison of the structure of compound **3** with that of compound **4**, it could be seen that methylation at the 4'-hydroxyl group of **4** to yield the corresponding methyl ether **3** resulted in loss of activity. Free 5- and 7-hydroxyl groups as well as the lack of a 6-methoxyl group seemed to increase biological activity. However, the existing data did not permit conclusion of the relatively high antimycobacterial activity of compound **1**.

Cytotoxicity

Compounds **1** and **3-8** were evaluated for cytotoxicity against KB, BC and NCI-H187 cells (Table II). All the tested compounds were inactive against the KB cells. Only compound **8** was active against the BC cells with the weak MIC value of 38.4 μM. Compounds **7** and **8** exhibited moderate activity against the NCI-H187 cells with the MIC values of 24.6 and 19.2 μM, respectively. It was noteworthy that among the three classes of flavonoids tested only the flavones were active against some cancer cell lines.

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C-25 epimeric 26-haloponasterone A: Synthesis, absolute configuration and moulting activity

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Abstract

A convenient synthesis of inokosterone has been accomplished. Inokosterone exists as two C-25 epimers, which could be separated from each other through their diacetonide derivatives. The absolute configuration of these compounds was determined. Two C-25 epimers of 26-chloroponasterone A were synthesized from the respective C-25 epimeric inokosterone. Two epimeric 26-bromo and 26-iodoponasterone A compounds were also synthesized. Moulting activity of these compounds was evaluated using the *Musca* bioassay, and it was found that the (25*S*)-26-halo analogues were more active than the corresponding (25*R*)-26-halo analogues. Among the 25*S* series, an increase in activity with an increase in size of the halogen atom was observed, indicating that the steric factor was more important than the electronic factor in binding of these ecdysteroid analogues to the receptor. On the other hand, a decrease in activity with an increase in size of the halogen atom was noted in the 25*R* series, suggesting that the steric factor was less important than the electronic factor. The results indicated that the configuration at C-25 and the substituent at C-26 have significant influences on the interaction of ecdysteroids with their receptor.

Keywords: Ecdysteroid; Inokosterone; 26-Haloponasterone A; Synthesis; Moulting activity; *Musca* bioassay

1. Introduction

Ecdysteroids are insect moulting hormones found in both invertebrates and plant species [1–3]. The established function of ecdysteroids in insects is the regulation of moulting. Other hormonal functions include regeneration, metamorphosis, reproduction, and differentiation [1,3]. 20-Hydroxyecdysone (**1**) is the most abundant representative of this class of compounds. Essential features contributing to moulting hormone activity include an A/B *cis*-ring junction, a 6-keto-7-ene function, a free 14α-hydroxyl group, and a full sterol side chain [4]. The number, location, and stereochemistry of hydroxyl groups in the molecule are also responsible for high activity of ecdysteroids.

In our studies on structure–activity relationships of ecdysteroids, we recently demonstrated that while abutasterone (**2**) and its C-24 epimer 24-epi-abutasterone (**3**) exhibited comparable activity, they were less active than compound **1** [5]. On the other hand, pterosterone (**4**), the C-25 deoxy analogue of **2**, was more active than compound **3**, while 24-epi-pterosterone (**5**) was much less active than its C-24 epimer compound **4** [6]. Also, in our recent study, we discovered that 20,26-dihydroxyecdysone (**6**) isolated from a number of *Vitex* plants existed as two C-25 epimers, and the two compounds could be separated from each other by HPLC. The shorter retention time component in reversed-phase HPLC was approximately two-fold less active than its epimers [7]. It was therefore logical to study moulting hormone activity of the C-25 deoxy analogue of **6** inokosterone (**7**). Compound **7** was a minor ecdysteroid isolated from *Achyranthes fauriei* [8]. It was also found in crustaceans [9]. This ecdysteroid existed as a 1:2 mixture of two C-25 epimers, but separation of these epimers was not accomplished [10].

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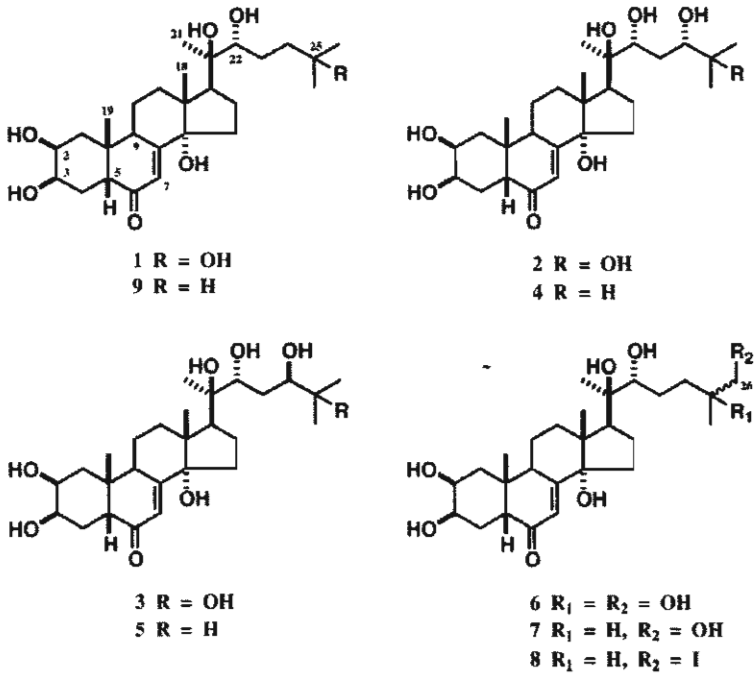


Fig. 1. Structure of ecdysteroids.

It has been reported that 26-iodoponasterone A (**8**) (Fig. 1) was 160 times as active as compound **1** in the in vitro Kc cell bioassay [11]. The unusually high activity of this ecdysteroid analogue has attracted our attention to investigate the in vivo moulting activity of 26-halo analogues. Since inokosterone consists of two C-25 epimers, it might show different binding of the ecdysteroid side chain to the receptor. It was therefore necessary to separate and evaluate both epimers of inokosterone, and the absolute stereochemistry at C-25 should be known. This report deals with the synthesis and determination of the absolute configuration of the two C-25 epimers of inokosterone from which the 26-halo analogues of ponasterone A (**9**) were synthesized. The moulting activity of the halo analogues was then evaluated.

2. Experimental

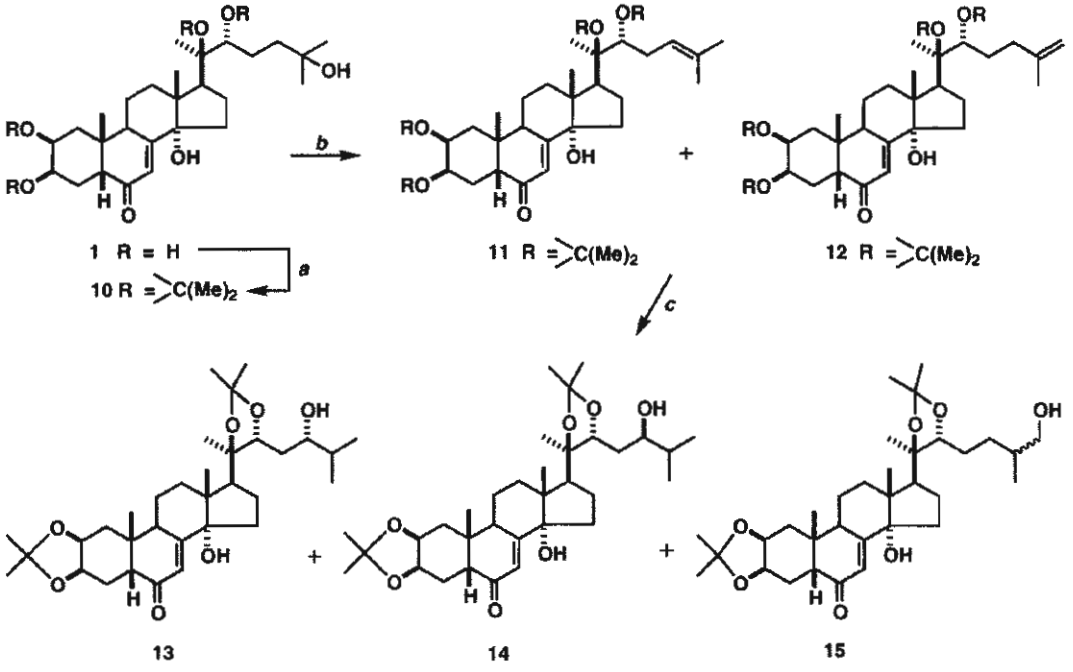
2.1. General methods

^1H and ^{13}C NMR were recorded on a Bruker AVANCE 400 spectrometer operating at 400 and 100 MHz, respectively. The chemical shifts (δ) are reported in ppm, and coupling constants (J) are given in Hz. For the spectra taken in C_5D_5N and $CDCl_3$, the residual nondeuterated solvent signals at δ 8.71 and 7.24 and the solvent signals at δ 149.90 and 77.00 were used as references for ^1H and ^{13}C spectra, respectively. FABMS and ESMS were measured with a Finnigan MAT 90 and a Bruker Esquire-LC mass spectrometers, respectively. Unless otherwise indicated, Merck silica gel 60 (finer than 0.063 mm) was used for column chromatography. TLC was performed on Merck silica gel 60 F_{254} precoated plates. The eluting solvent system for column chromatography used throughout the experiments was CH_2Cl_2–MeOH, with an increasing percentage of the more polar solvent. Spots on TLC were visualized under UV light and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating [12].

2.2. Synthesis of inokosterone 2,3;20,22-diacetonide

Diacetonide **10** (696 mg), which was prepared from compound **1** by a procedure previously reported in the literature [13], was dissolved in pyridine (5 ml), and the solution was kept at about 5 °C for 5 min. Mesyl chloride (1 ml) was added, and the reaction mixture stirred for 5 min. 4-Dimethylaminopyridine (DMAP) (25 mg) was added, and stirring was continued at 5 °C for 30 min. Then, the reaction mixture was slowly allowed to warm up to ambient temperature, during which time the progress of the reaction was monitored by TLC. One percentage of aqueous $NaHCO_3$ solution was added, and the mixture extracted with $CHCl_3$ (3 × 40 ml). The combined $CHCl_3$ layer was washed with water, dried, and evaporated to dryness. The crude mixture was chromatographed using $CHCl_3$–MeOH (99:1) to afford a 3:2 mixture of compounds **11** and **12** (472 mg, 70%). IR ν_{max} 3470, 2975, 1658, 1445, 1373, 1243, 1215, 1057, 884 cm^{-1}; ^1H NMR data were consistent with those reported previously [5]; FAB MS (−ve) *m/z* (% relative intensity) 541 $[M-H]^-$.

BH_3–THF complex (ca 1 M in THF, 0.5 ml) was added in portions to a solution of compounds **11** and **12** mixture



Scheme 1. Reagents and conditions: (a) MeCOMe/TsOH; (b) reaction from **10**; MsCl/pyridine/DMAP; (c) (i) BH_3–THF, (ii) H_2O_2/NaOH.

(ratio 3:2) (295 mg) in THF (5 ml) while stirring, and the reaction mixture was stirred for 2 h. Hydrogen peroxide solution (35%, 0.5 ml) and 1N NaOH (0.5 ml) were then added, and stirring was continued for 5 min. Water (200 ml) was added, and the mixture extracted with EtOAc (3× 50 ml). The combined organic layer was washed with water (100 ml), dried over anhydrous Na_2SO_4, and evaporated to dryness. The crude product was carefully chromatographed with $CHCl_3$–MeOH as eluting solvent to yield compound **13** (67 mg, 22%). Compounds **14**, **15** "epimer 1", and **15** "epimer 2" were eluted, but found to be contaminated by each other. Repeated column chromatography yielded pure compounds **14** (67 mg, 22%), **15** "epimer 1" (64 mg, 21%), and **15** "epimer 2" (61 mg, 20%) (Scheme 1).

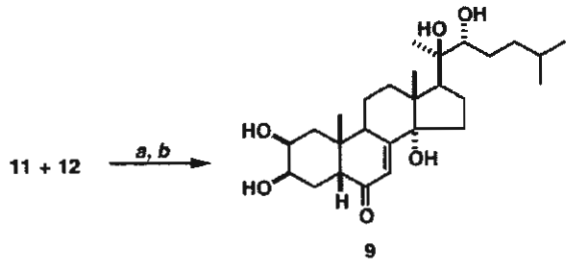
Compound **13**: IR ν_{max} 3535, 3458, 2960, 1642, 1373, 1245, 1225, 1170, 1060, 881 cm^{-1}; ^1H NMR data are given in Table 1; HR FABMS (−ve) *m/z* 559.3639 $[M-H]^-$. $C_{33}H_{52}O_7$-H requires 559.3634.

Compound **14**: IR ν_{max} 3468, 2961, 2870, 1660, 1466, 1371, 1244, 1217, 1169, 1058, 878 cm^{-1}; ^1H NMR data are

Table 1
^1H NMR data of compounds **13**, **14**, **15** "epimer 1", **15** "epimer 2", **20**, and **21**

Proton	13[a]	14[a]	15[a] "epimer 1"	15[a] "epimer 2"	20[b]	21[b]
2	4.20 (m)	4.20 (m)	4.20 (m)	4.20 (m)	4.17 (m)	4.17 (m)
3	4.25 (brs)	4.25 (brs)	4.25 (brs)	4.25 (brs)	4.23 (m)	4.23 (m)
5	2.34 (dd, 12.6, 4.7)	2.33 (dd, 12.5, 4.8)	2.33 (dd, 12.5, 4.5)	2.33 (dd, 12.6, 4.7)	3.01 (dd, 13.2, 3.8)	3.01 (dd, 13.2, 3.8)
7	5.81 (d, 2.1)	5.80 (d, 2.4)	5.80 (d, 2.1)	5.80 (d, 2.1)	6.25 (d, 2.4)	6.25 (d, 2.4)
9	2.78 (m)	2.79 (m)	2.79 (m)	2.78 (m)	3.59 (m)	3.59 (m)
17	2.17 (dd, 8.5, 7.9)	2.18 (dd, 9.4, 7.9)	2.19 (dd, 9.7, 9.4)	2.18 (dd, 9.4, 8.5)	2.95 (t, 9.1)	2.94 (d, 9.1)
22	3.80 (dd, 9.7, 2.7)	3.96 (dd, 10.3, 1.5)	3.61 (dd, 6.7, 5.8)	3.62 (br d, 9.1)	3.85 (br d, *ca* 9)	3.86 (br d, 9.7)
24	3.53 (m)	3.54 (m)				
26	–	–	3.45 (dd, 10.6, 5.8), 3.48 (dd, 10.6, 5.7)	3.44 (dd, 10.3, 6.1), 3.48 (dd, 10.3, 6.4)	3.65 (dd, 10.3, 6.4), 3.77 (dd, 10.3, 5.5)	3.64 (dd, 10.3, 6.1), 3.72 (dd, 10.3, 5.8)
18-Me	0.77 (s)	0.77 (s)	0.769 (s)	0.767 (s)	1.215 (s)	1.217 (s)
19-Me	0.96 (s)	0.96 (s)	0.960 (s)	0.959 (s)	1.064 (s)	1.067 (s)
21-Me	1.14 (s)	1.12 (s)	1.119 (s)	1.115 (s)	1.563 (s)	1.577 (s)
26-Me	0.93 (d, 6.7)	0.91 (d, 6.7)	–	–	–	–
27-Me	0.93 (d, 6.7)	0.93 (d, 6.7)	0.924 (d, 6.7)	0.912 (d, 6.7)	1.036 (d, 6.7)	1.040 (d, 6.1)
>C(Me)$_2$	1.31, 1.32, 1.39, 1.47 (each s)	1.30, 1.31, 1.38, 1.46 (each s)	1.295, 1.307, 1.381, 1.468 (each s)	1.297, 1.307, 1.384, 1.468 (each s)	–	–

[a] Recorded in $CDCl_3$.
[b] Recorded in C_5D_5N.



Scheme 2. Reagents and conditions: (a) H_2/Pd-C, EtOH; (b) 70% AcOH.

given in Table 1; HR FABMS (−ve) *m/z* 559.3634 $[M-H]^-$. $C_{33}H_{52}O_7$-H requires 559.3634.

Compound **15** "epimer 1": IR ν_{max} 3451, 2938, 1658, 1647, 1371, 1244, 1217, 1170, 1108, 1059, 883 cm^{-1}; ^1H NMR data are given in Table 1; HRFABMS (−ve) *m/z* 559.3632 $[M-H]^-$. $C_{33}H_{52}O_7$-H requires 559.3634.

Compound **15** "epimer 2": IR ν_{max} 3436, 2937, 2875, 1658, 1451, 1371, 1244, 1217, 1170, 1108, 1058, 883 cm^{-1}; ^1H NMR data are given in Table 1; HR FABMS (−ve) *m/z* 559.3632 $[M-H]^-$. $C_{33}H_{52}O_7$-H requires 559.3634.

2.3. Synthesis of ponasterone A

A mixture of compounds **11** and **12** (109 mg) in EtOH (2 ml) was subjected to catalytic hydrogenation at atmospheric pressure for 20 min with 5% Pd-C (100 mg) as a catalyst. The mixture was filtered through a short alumina column, and the residue was washed with EtOH. The filtered solution was evaporated to dryness, and the crude product (116 mg) was subjected to acetonide deprotection in 70% AcOH (4 ml) for 4 days. The reaction mixture was poured into water (100 ml) and extracted with EtOAc (3× 30 ml). The EtOAc layer was washed with water (100 ml), dried over anhydrous Na_2SO_4, and evaporated. The crude product was carefully chromatographed with $CHCl_3$–MeOH as eluting solvent to yield compound 9 (74 mg, 79%) (Scheme 2).

Compound **9**: Mp: 256–258 °C; IR ν_{max} 3400, 2956, 1641, 1459, 1380, 1108, 1052 cm^{-1}; ^1H NMR (C_5D_5N): δ 0.81 (d, J = 6.1 Hz, 3H, 26-Me), 0.82 (d, J = 6.1 Hz, 3H, 27-Me), 1.07 (s, 3H, 19-Me), 1.23 (s, 3H, 18-Me), 1.57 (s, 3H, 21-Me), 2.93 (t, J = 9.1 Hz, 1H, H-17), 3.02 (dd, J = 13.2, 3.8 Hz, 1H, H-5), 3.60 (m, 1H, H-9), 3.80 (br d, J = 10.4 Hz, 1H, H22), 4.16 (m, 1H, H2), 4.23 (br s, 1H, H3), 6.26 (d, J = 2.4 Hz, 1H, H-7).

2.4. Determination of C-25 absolute configuration of inokosterone

The two epimers of inokosterone 2,3;20,22-diacetonide (**15** "epimer 1" and **15** "epimer 2") (2 mg each) were separately esterified with (+)- and (−)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chlorides (MTPA chlorides) (each 10 μl) in dry pyridine (200 μl). The reaction was monitored using TLC. Typically, the reaction was completed within 10 min. 1 M HCl (20 ml) was poured into the reaction mixture and the solution was extracted with CH_2Cl_2 (3× 10 ml). The combined organic layer was sequentially washed with 10% $NaHCO_3$ and water and then dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and the ^1H NMR spectra of the Mosher's esters **16**, **17**, **18**, and **19** were recorded (Table 2).

Table 2
Chemical shifts of C-26 methylene protons of Mosher's ester

Compound	MTPA	$\delta H_{26(a)}$ (ppm)	$\delta H_{26(b)}$ (ppm)	$\Delta\delta$ (ppm)
16	(+)-(*S*)	4.10	4.23	0.13
17	(−)-(*R*)	4.03	4.29	0.26
18	(+)-(*S*)	4.08	4.25	0.17
19	(−)-(*R*)	4.17	4.22	0.05

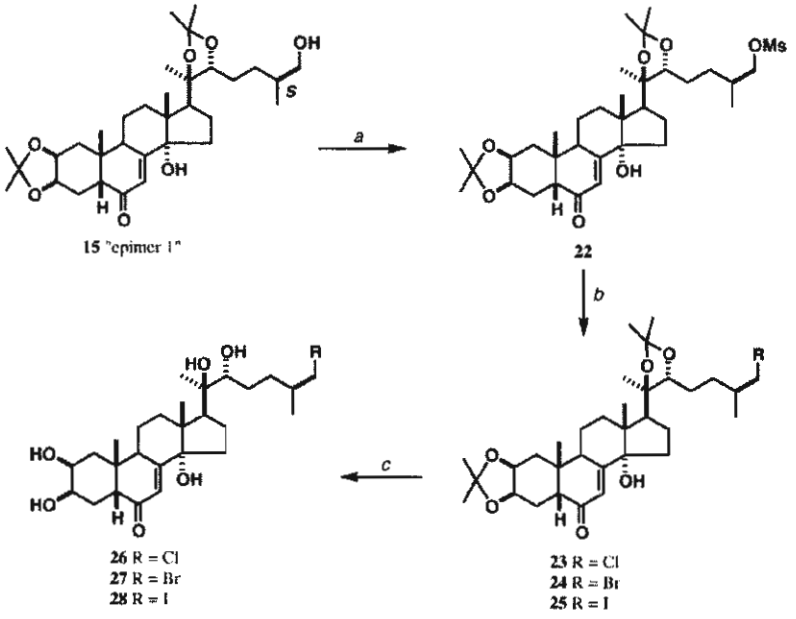
2.5. Acetonide deprotection of compound 15

Seventy percentage of aqueous acetic acid (1 ml) was added to a solution of compound **15** "epimer 1" (10 mg) in dioxane (1 ml), and the mixture was stirred for 3 days [13]. Water (50 ml) was added, and the mixture was thoroughly extracted with *n*-butanol until no product was detected in the aqueous phase. The solvent was evaporated by co-distillation with water. Column chromatography yielded inokosterone "epimer 1", 25(*S*)-inokosterone (**20**) (6.4 mg, 75%). IR ν_{max} 3386, 2937, 1640, 1383, 1049, 872 cm^{-1}; ^1H NMR data given in Table 1; HR FABMS (−ve) *m/z* 479.3004 $[M-H]^-$. $C_{27}H_{44}O_7$-H requires 479.3008.

Compound **15** "epimer 2" (9 mg) was subjected to deacetonation in the same manner described for compound **15** "epimer 1" to afford 25(*R*)-inokosterone (**21**) (5.9 mg, 77 %). IR ν_{max} 3387, 2943, 1641, 1384, 1052 cm^{-1}; ^1H NMR data given in Table 1; HR FABMS (−ve) *m/z* 479.3001 $[M-H]^-$. $C_{27}H_{44}O_7$-H requires 479.3008.

2.6. Synthesis of 26-halo analogues of inokosterone

General procedure. The diacetonide **15** "epimer 1" (60 mg) was dissolved in pyridine (2 ml), and mesyl chloride (0.1 ml) was added. The mixture was stirred at ambient temperature for 10 min, and water was then added. The solution was extracted with $CHCl_3$ (3× 30 ml); the organic phase was washed with water, dried over anhydrous Na_2SO_4, and the solvent was evaporated to dryness. Column chromatography of the crude product on silica gel afforded the diacetonide mesylate **22** (spectroscopic data are not shown), which was subjected to chloride displacement by stirring a solution of **22** and NaCl in dimethylformamide for 4 days. After the usual workup, the product was subjected to deacetonation with 70% acetic acid and chromatographed to give (25*S*)-26-chloroponasterone A (**26**) (Scheme 3). The overall yield from (25*S*)-inokosterone 2,3;20,22-diacetonide (**15** epimer 1) was 54%. Following the same procedure, but with different halide



Scheme 3. Reagents and conditions: (a) MsCl/pyridine; (b) NaCl/DMF (use KBr and KI instead of NaCl for **24** and **25**, respectively); (c) 70 % AcOH/dioxane.

salts (KBr and KI), the (25*S*)-26-bromo **27** and (25*S*)-26-iodo **28** analogues were synthesized in 59 and 32% overall yields, respectively. The (25*R*)-26-halo analogues **29**–**31** were similarly synthesized in 34, 42, and 32% overall yields, respectively.

2.6.1. (25S)-26-chloroponasterone A (26)

Mp: 186–187 °C; IR, ν_{max} (cm^{-1}): 3420, 2956, 1645, 1383, 1053; ^1H NMR, see Table 3; ESMS (+ve), *m/z* (% rel. intensity): 521 $[M+Na]^+$ (100), 523 $[M+Na]^+$ (38). HRFABMS (−ve), *m/z*: 497.2667. $C_{27}H_{43}O_6Cl$-H requires 497.2670.

2.6.2. (25S)-26-bromoponasterone A (27)

Amorphous; IR, ν_{max} (cm^{-1}): 3395, 2929, 1642, 1447, 1382, 1055; ^1H NMR see Table 3; ESMS (+ve), *m/z* (% rel. intensity): 565 $[M+Na]^+$ (83), 567 $[M+Na]^+$ (82).

2.6.3. (25S)-26-iodoponasterone A (28)

Amorphous; IR, ν_{max} (cm^{-1}): 3422, 2928, 1639, 1450, 1383, 1053; ^1H NMR see Table 3; ESMS (+ve), *m/z* (% rel. intensity): 613 $[M+Na]^+$ (100). HRFABMS (−ve), *m/z*: 589.2026. $C_{27}H_{43}O_6I$-H requires 589.2025.

2.6.4. (25R)-26-chloroponasterone A (29)

Mp: 185–186 °C; IR, ν_{max} (cm^{-1}): 3421, 2949, 1638, 1384, 1056; ^1H NMR see Table 3; ESMS (+ve), *m/z* (% rel. intensity): 521 $[M+Na]^+$ (100), 523 $[M+Na]^+$ (32). HRFABMS (−ve), *m/z*: 497.2673. $C_{27}H_{43}O_6Cl$-H requires 497.2670.

2.6.5. (25R)-26-bromoponasterone A (30)

Amorphous; IR, ν_{max} (cm^{-1}): 3395, 2929, 1642, 1447, 1382, 1055; ^1H NMR see Table 3; ESMS (+ve), *m/z* (% rel. intensity): 565 $[M+Na]^+$ (70), 567 $[M+Na]^+$ (67).

Table 3
^1H NMR data of 26-haloponasterone A recorded in C_5D_5N

Proton	26	27	28	29	30	31
2	4.17 (m)	4.16 (m)	4.17 (m)	4.17 (m)	4.18 (m)	4.17 (m)
3	4.24 (brs)	4.22 (brs)	4.23 (brs)	4.23 (brs)	4.24 (brs)	4.23 (brs)
5	3.02 (dd, 13.1, 3.8)	3.00 (dd, 13.1, 3.7)	3.01 (dd, 13.3, 3.8)	3.02 (dd, 13.2, 3.8)	3.02 (dd, 13.2, 3.0)	3.02 (dd, 13.1, 3.0)
7	6.27 (d, 2.2)	6.24 (d, 2.4)	6.25 (d, 2.4)	6.27 (d, 2.4)	6.26 (brs)	6.26 (d, 2.1)
9	3.60 (m)	3.58 (m)	3.59 (m)	3.60 (m)	3.60 (m)	3.60 (m)
17	2.92 (t, 9.1)	2.90 (t, 9.0)	2.91 (t, 9.0)	2.91 (t, 9.1)	2.91 (t, 9.0)	2.91 (t, 9.0)
22	3.79 (dd, 10.2, 3.3)	3.78 (brd, 9.8)	3.78 (brd, 9.2)	3.80 (dd, 9.6, 3.3)	3.80 (brd, 9.9)	3.79 (brd, 7.9)
26a	3.51 (dd, 10.6, 4.7)	3.42 (dd, 9.8, 4.5)	3.24 (dd, 9.6, 4.1)	3.47 (dd, 10.6, 4.4)	3.41 (dd, 9.7, 3.6)	3.20 (dd, 9.5, 4.1)
26b	3.40 (dd, 10.6, 6.4)	3.32 (dd, 9.8, 6.3)	3.12 (dd, 9.6, 6.0)	3.38 (dd, 10.6, 5.8)	3.32 (dd, 9.5, 5.8)	3.11 (dd, 9.3, 6.3)
18	1.23 (s)	1.20 (s)	1.22 (s)	1.22 (s)	1.22 (s)	1.22 (s)
19	1.07 (s)	1.05 (s)	1.06 (s)	1.07 (s)	1.07 (s)	1.07 (s)
21	1.57 (s)	1.56 (s)	1.57 (s)	1.58 (s)	1.58 (s)	1.57 (s)
27	0.91 (d, 6.6)	0.90 (d, 6.6)	0.87 (d, 6.4)	0.92 (d, 6.3)	0.94 (d, 5.7)	0.88 (d, 6.4)

Table 4
EC_{50} of ecdysteroids

Compound	EC_{50} (M)[a]
20	3.4×10^{-5}
21	7.4×10^{-5}
26	7.6×10^{-5}
27	5.0×10^{-5}
28	3.7×10^{-5}
29	1.1×10^{-4}
30	1.3×10^{-4}
31	5.4×10^{-4}
Ponasterone A (**9**)	8.7×10^{-6}
20-Hydroxyecdysone (**1**)	1.6×10^{-5}

[a] Molar concentration of ecdysteroid solution (3 μl per larva) required to effect puparium formation with 50% effectiveness [15].

HRFABMS (−ve), *m/z*: 543.2314. $C_{27}H_{43}O_6Br$-H requires 543.2321.

2.6.6. (25R)-26-iodoponasterone A (31)

Amorphous; IR, ν_{max} (cm^{-1}): 3431, 2929, 1645, 1380, 1063; ^1H NMR see Table 3; ESMS (+ve), *m/z* (% rel. intensity): 613 $[M+Na]^+$ (100).

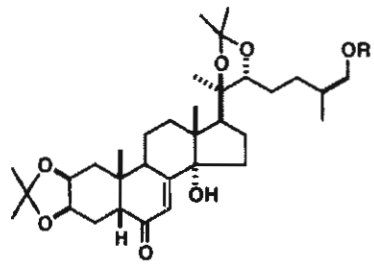
2.7. Bioassay

The *Musca* assay was performed according to Kaplanis et al. [14]. Briefly, mature *Musca domestica* larvae were ligated on the seventh or eighth segment, and those that had formed puparia at their anterior ends, but had abdominal compartments that remained in the larval stage, were used. A 1 mg portion of ecdysteroid was dissolved in 24–48 μl of absolute ethanol, and the solution was subsequently diluted with distilled water to a volume of 300 μl. The resulting ecdysteroid solution, the initial concentration of which was 10 μg/3 μl, was diluted with distilled water to appropriate concentrations depending on the activity of each ecdysteroid. Three microliters of each ecdysteroid concentration were injected into the abdominal region of the ligated insect. Ten ligated larvae were used for each ecdysteroid concentration. The results were scored according to Ohtaki et al. [15], and EC_{50}s were determined by plotting concentrations (calculated in molar) against % effectiveness of puparium formation (see Table 4).

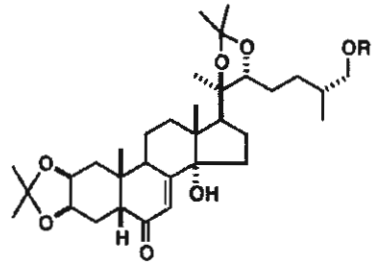
3. Results and discussion

3.1. Synthesis of inokosterone 2,3:20,22-diacetonide

Starting from the readily available ecdysteroid 1 isolated from *V. glabrata* [16], a 3:2 mixture of the olefin diacetonides **11** and **12** was obtained from diacetonide **10** [13] (see Scheme 1). Two epimeric inokosterone 2,3;20,22-diacetonides were synthesized by the method of Yingyongnarongkul and Suksamrarn [17] with some modification.



16 R = (+)-MTPA
17 R = (-)-MTPA



18 R = (+)-MTPA
19 R = (-)-MTPA

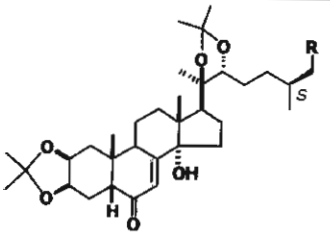
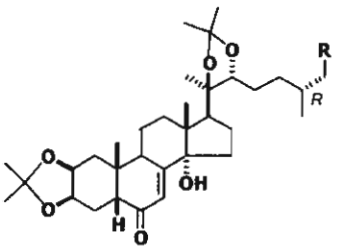
Fig. 2. Mosher's ester derivatives of inokosterone.

Thus, a mixture of compounds **11** and **12** was subjected to hydroboration using diborane–THF complex, followed by treatment with alkaline hydrogen peroxide to afford, after careful column chromatography, pterosterone 2,3:20,22-diacetonide (**13**), 24-*epi*-pterosterone 2,3:20:22-diacetonide (**14**), 25(*S*)-inokosterone 2,3:20:22-diacetonide (**15** "epimer 1"), and 25(*R*)-inokosterone 2,3:20,22-diacetonide (15 "epimer 2") in 22, 22, 21, and 20% yield, respectively. ^1H NMR data (Table 1) were in agreement with the structures.

3.2. Determination of C-25 configuration of inokosterone

It has been shown that (25*R*)- and (25*S*)-26-hydroxy steroids can be differentiated by the ^1H NMR of their (+)- and (−)-MTPA esters. In the spectra of the (+)-MTPA ester, the 26-methylene protons of the 25*S* isomer appear as signals resonating much closer than in the corresponding 25*R* isomer, and the reverse is observed with the (−)-MTPA ester such that the resonance signals in the 25*R* isomer are closer than in the 25*S* isomer [18]. Thus, inokosterone 2,3;20,22-diacetonide **15** (epimer 1) and **15** (epimer 2) were separately treated with (+)- and (−)-MTPA-Cl to afford the esters **16**, **17**, **18**, and **19**, respectively (Fig. 2). The chemical shift difference ($\Delta\delta$) of the 26-methylene protons is shown in Table 2. Comparison of the chemical shift difference ($\Delta\delta$ 0.13 ppm) of **16** observed at δ 4.10 and 4.23 with that ($\Delta\delta$ 0.26 ppm) of **17** at δ 4.03 and 4.29 indicated the 25*S* configuration of

Table 5
H-26 chemical shift of 26-haloponasterone A 2,3;20,22-diacetonide derivatives in $CDCl_3$

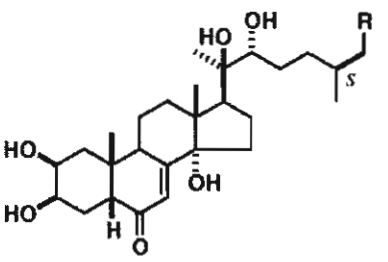
Structure	R	$\delta H_{26(a)}$ (ppm)	$\delta H_{26(b)}$ (ppm)	$\Delta\delta H_{26(a)-26(b)}$ (ppm)
	Cl	3.49	3.39	0.10
	Br	3.41	3.30	0.11
	I	3.24	3.11	0.13
	Cl	3.47	3.43	0.04
	Br	3.39	3.35	0.04
	I	3.22	3.16	0.06

15 (epimer 1). By the same analogy, the chemical shift difference in (+)- and (−)-MTPA esters (**18** and **19**) revealed that the configuration at C-25 of **15** (epimer 2) was *R*. Since chemical reactions involved in the synthesis of the 26-halo analogues **26–31** did not affect the C-25 configurations of these compounds, the absolute configurations of these compounds were the same as those of the starting ecdysteroid **15** epimers.

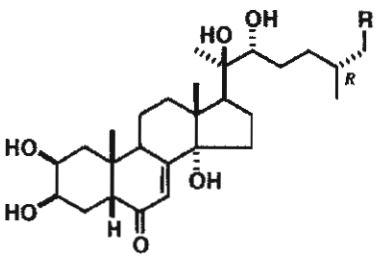
It should be noted that the H_{26a} and H_{26b} signals of (25*S*)-26-haloponasterone 2,3;20,22-diacetonide recorded in $CDCl_3$ were more far apart ($\Delta\delta$ 0.10–0.13 ppm) than those of the corresponding (25*R*)-isomers ($\Delta\delta$ 0.03–0.06 ppm) (Table 5). This finding helped with the estimation of the stereochemistry at C-25 of the 25-deoxy-26-halo-ecdysteroids. Such a difference was also observed in the corresponding deacetonation products **26–31** recorded in C_5D_5N.

3.3. Synthesis of 26-halo analogues of inokosterone

The synthesis of (25*S*)-26-chloro (**26**), (25*S*)-26-bromo (**27**), and (25*S*)-26-iodo (**28**) analogues (Fig. 3) was performed as follows: treatment of the pyridine solution of diacetonide **15** "epimer 1" with mesyl chloride gave the corresponding mesylate **22** in high yield. Substitution of the OMs group with appropriate halide ions in dimethylformamide at room temperature would have given the (25*S*)-26-haloponasterone A 2,3;20,22-diacetonede without intact C-25 stereochemistry. Thus, treatment of (25*S*)-26-mesyl ponasterone A 2,3;20,22-diacetonide (**22**) with NaCl, KBr, and KI afforded (25*S*)-26-chloro-ponasterone A 2,3;20,22-diacetonede (**23**), (25*S*)-26-bromo-ponasterone A 2,3;20,22-diacetonede (**24**), and (25*S*)-26-chloro-ponasterone A 2,3;20,22-diacetonede (**25**), respectively (see Scheme 3). The desired products (**26–28**)



26 R = Cl
27 R = Br
28 R = I



29 R = Cl
30 R = Br
31 R = I

Fig. 3. Structure of two C-25 epimeric 26-haloponasterone A compounds.

were obtained after acetonide deprotection with 70% acetic acid [13]. Compounds **26**, **27**, and **28** were characterized by ESMS, HRFABMS, and ^{1}H NMR spectroscopy (Table 3). The (25*R*)-26-chloro (**29**), (25*R*)-26-bromo (**30**) and (25*R*)-26-iodo (**31**) analogues (Fig. 3) were also prepared and characterized in the same manner as their epimers.

3.4. Synthesis of 25(S)-inokosterone (20) and 25(R)-inokosterone (21)

In order to compare the effect of position and orientation of the hydroxyl group of the C-26 sterol side chain on moulting activity, compounds **20** and **21** were synthesized. Acetonide deprotection of 25(*S*)-inokosterone 2,3;20,22-diacetonide (**15** "epimer 1") and 25(*R*)-inokosterone 2,3;20,22-diacetonide (**15** "epimer 2") with 70% AcOH [13] afforded compounds **20** and **21** in 75 and 77% yield, respectively.

3.5. Synthesis of ponasterone A (9)

In order to obtain more information to explain the moulting activity of ecdysteroids without any substituents on the C-25 and C-26 positions, ponasterone A (**9**) was synthesized. With a 3:2 mixture of olefin diacetonides **11** and **12** in hand, catalytic hydrogenation followed by acetonide deprotection afforded ponasterone A (**9**) in 79% yield over two steps. ^{1}H NMR data were in agreement with the structures.

3.6. Biological activity of the 26-halo analogues

(25*S*)-inokosterone (**20**) exhibited approximately two-fold higher moulting hormone activity than its C-25 epimer (25*R*)-inokosterone (**21**) in the *Musca* bioassay. The 26-halo analogues also followed this trend. The 25*S* isomers of each halide (i.e., compounds **26**, **27**, and **28**) were more active than the corresponding 25*R* isomers (i.e., compounds **29**, **30**, and **31**, respectively) (see Table 4). However, they were less active than 20-hydroxyecdysone (**1**). It was noteworthy that all of the 25*S* isomers were more active than the 25*R* isomers. Interestingly, among the 25*S* series, an increase in activity with an increase in the size of the halogen atom was observed, thus, indicating that with binding of these ecdysteroid analogues to the receptor, the steric factor was more important than the electronic factor. On the other hand, a decrease in activity with increasing size of the halogen atom was observed in the 25*R* series. In the latter case, the steric factor might be less important than the electronic factor. These results clearly indicated that the configuration at C-25 and the substituent at C-26 have significant influences on the interaction of ecdysteroids with their receptor.

It was thus evident that introduction of the C-26 hydroxyl group or halide atom to ponasterone A (**9**) (Fig. 1) in both 25*S* and 25*R* orientation resulted in a significant decrease in activity. It was noteworthy that introduction of the C-26 hydroxyl group to ponasterone A (i.e., compounds **20** and **21**) caused a greater decrease in activity than did the C-25 hydroxyl group (i.e., 20-hydroxyecdysone (**1**)). Our finding allowed us to gain insight into the binding of ecdysteroids, especially the extremity of the side chain, to the receptor. It was noteworthy that in the case of 26-iodoponasterone A (**3**), which was exceptionally active in the in vitro Kc cell assay, the two C-25 epimers, (i.e., compounds **28** and **31**) were less active than the corresponding parent inokosterones **20** and **21** in the in vivo *Musca* assay. The existing data did not permit the explanation of such large differences in molting activities seen with these two assay systems.

Acknowledgements

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Cytotoxic Prenylated Xanthones from the Young Fruit of *Garcinia mangostana*

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Three new prenylated xanthones, mangostenones C (1), D (2), and E (3), together with 16 known xanthones 4—19, were isolated from the young fruit (7-week maturity stage) of *Garcinia mangostana*. The structural elucidation of the new compounds was mainly established on the basis of 1D and 2D NMR and HR-MS spectroscopic analysis. Compound 1 showed cytotoxic properties against three human cancer cell lines, epidermoid carcinoma of the mouth (KB), breast cancer (BC-1), and small cell lung cancer (NCI-H187), with IC_{50} values of 2.8, 3.53, and 3.72 μg/ml, respectively. Among the isolates, α-mangostin (12), the major metabolite, exhibited the most potent effects against the BC-1 cells with an IC_{50} value of 0.92 μg/ml, an activity greater than that of the standard drug ellipticine (IC_{50}=1.46 μg/ml). Compound 12 also showed the highest activity against KB cells, while gartanin (10) displayed the strongest activity against the NCI-H187 cells at the respective IC_{50} values of 2.08 μg/ml and 1.08 μg/ml.

Key words xanthone; *Garcinia mangostana*; Clusiaceae; mangostenone C; mangostenone D; mangostenone E; cytotoxicity

Biological studies on the xanthones obtained from the fruit of the tropical tree *Garcinia mangostana* L. (Clusiaceae), regarded as "the queen of fruit," have demonstrated interesting biological activities.[1,2] Studies have been conducted to examine the anticancer properties of the extracts or xanthones obtained from the fruit hulls of this plant species against colon preneoplastic lesions,[3] DNA topoisomerases I and II,[4] human leukemia (HL60, K562, NB4, U937, P3HR1, and Raji),[5,6] hepatoma (HCC36, TONG, HA22T, Hep 3B, HEpG2, and SK-HEp-1), lung (NCI-Hut 125, CH27-LC-1, H2981, and Calu-1), and gastric carcinomas (AZ521, NUGC-3, KATO-III, and AGS),[7] and human breast cancer SKBR3 cells.[8,9] Our interest has been focused on the isolation of structurally interesting prenylated xanthones and the biological activities of intermediates at various stages of fruit maturity. In our previous study, the isolation of 17 xanthones including three new xanthones, mangostenol and mangostenones A and B, from the green fruit (14-week maturity stage) of this plant and their antituberculosis activity were already described.[10,11] In a continuation of this work, the EtOAc-soluble extract obtained from the young fruit (7-week maturity stage) of *G. mangostana* was subjected to a chemical investigation leading to the isolation and structural elucidation of three new prenylated xanthones, mangostenones C (1), D (2), and E (3), in addition to 16 previously reported xanthones, thwaitesixanthone (4),[12] demethylcalabaxanthone (5),[11] garcinone B (6),[10] compound 7,[11] β-mangostin (8),[10] 8-desoxygartanin (9),[13] gartanin (10),[14] garcinone E (11),[15] the major metabolite α-mangostin (12),[10] mangostinone (13),[10] the second major metabolite γ-mangostin (14),[11] mangostanol (15),[10] mangostanin (16),[11] garcinone D (17),[11] garcinone C (18),[16] and the third major metabolite 11-hydroxy-1-isomangostin (19).[17] We here report on the structural elucidation of the three new compounds and the *in vitro* cytotoxic activities of xanthones **1**—**19** against three human cancer cell lines, epidermoid carcinoma of the mouth (KB), breast cancer (BC-1), and small cell lung cancer (NCI-H187).

Results and Discussion

The positive-ion mode HR-FAB-MS of compound **1** showed a pseudo molecular ion at *m/z* 443.1691 which corresponded to a molecular formula of $C_{24}H_{26}O_8$. The UV and IR spectra indicated that **1** has a xanthone skeleton. The ^{1}H-NMR spectrum of **1** (Table 1) exhibited the presence of two aromatic protons at δ 6.29 and 6.83, two phenolic hydroxyl groups at δ 6.38 and 13.92, a methoxyl at δ 3.81, and a prenyl group at δ 4.07, 5.23, 1.82, and 1.69. In the HMBC spectrum (Fig. 1), the methoxyl group at δ 3.81 showed a correlation with the C-7 (δ 143.0) and the hydroxyl proton at δ 6.38 correlated with C-6 and C-5. This methoxyl signal also exhibited NOE enhancement with H-16 at δ 4.07, H-17 at δ 5.23, and 6-OH in the NOESY spectrum (Fig. 1), confirming the placement of the methoxyl and the hydroxyl groups at C-7 and C-6, respectively. The two singlet methyls adjacent to an oxygen function (δ_H 1.40, δ_C 18.1 and δ_H 1.43, δ_C 23.7) and two oxygenated methine protons (δ_H 6.10, δ_C 82.8 and δ_H 5.29, δ_C 98.5) in the NMR data evidenced the presence of a 4-hydroxy-5-(1-hydroxy-1-methylethyl)dihydrofuran system in the side chain. The *cis* relationship of the two oxygenated methine protons was established on the basis of the coupling constant value of 6.1 Hz[12] in the ^{1}H-NMR spectrum. The correlation between these two methines and respective methyls observed in the NOESY spectrum in addition to the observation of the fragment ion at *m/z* 383 $[M-59]^+$ in the EI-MS confirmed that the 4-hydroxy-5-(1-hydroxy-1-methylethyl)dihydro furan moiety is preferable to the 4,5-dihydroxy-6,6-dimethyldihydropyran system for the partial structure of **1**. There remained the placement of the dihydrofuran ring. In the HMBC spectrum, the proton resonating at δ 5.29 (H-12) was correlated with an aromatic carbon at δ 167.0 (C-3) through ^3J, which implied that the

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Table 1. ^{1}H- (300 MHz) and ^{13}C-NMR (75 MHz) Data for Compounds **1** (in $CDCl_3$), **2** and **3** (in Acetone-d_6)[a]

Position	1		2		3	
	^{1}H (δ)	^{13}C (δ)	^{1}H (δ)	^{13}C (δ)	^{1}H (δ)	^{13}C (δ)
1		160.8		161.5		161.5
2		104.4[c]		110.8		111.1
3		167.0		162.6		163.3
4	6.29 s	88.1	6.38 s	93.0	6.40 s	93.2
4a		158.9		155.8		156.1[f]
5	6.83 s	101.6	6.73 s	101.3	6.84 s	102.9
6		154.7		154.0[e]		155.7[f]
7		143.0		139.8		145.5
8		137.1		122.9		137.0
8a		113.5		111.3		112.6
9		182.5		183.2		183.7
9a		105.6[c]		103.7		103.5
10a		155.7		153.9[e]		157.5
11	6.10 d (6.1)	82.8	3.34 d (6.9)	21.9	3.33 d (6.6)	21.9
12	5.29 d (6.1)	98.5	5.27 br t (6.9)	123.5	5.27 br t (6.6)	123.3
13		85.5		131.3		131.4
14	1.43 s[b]	23.7[d]	1.63 s	25.8	1.62 s	25.8
15	1.40 s[b]	18.1[d]	1.77 s	18.6	1.76 s	17.8
16	4.07 d (6.0)	26.5	3.47 t (6.6)	23.2	3.59 m	29.5
17	5.23 br t (6.0)	122.9	1.87 t (6.6)	33.2	3.69 m	79.7
18		132.3		75.2		73.2
19	1.69 s	25.7	1.34 s	26.4	1.29 s	26.1[g]
20	1.82 s	18.1				25.4[g]
1-OH	13.92 s		13.79 s		13.58 s	
3-OH			9.55 s			
6-OH	6.38 br s		8.79 s		9.86 br s	
7-OMe	3.81 s	62.0			3.85 s	60.8

a) δ in ppm, value in parentheses are coupling constant in Hz. b—g) Interchangeable within a column.

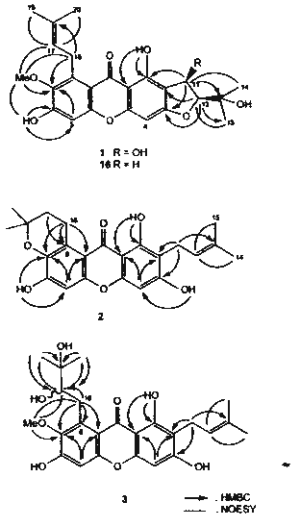

Fig. 1. Selected HMBC and NOESY Correlations for Compounds **1**—**3**

furan ring oxygen belongs to the hydroxyl group at C-3. The chemical shifts of C-11, C-12, and C-13 were in agreement with those reported for caloxanthone D.[18] Although this modification of a prenyl side chain is not common in *Garcinia*, it has been found in *Calophyllum*.[18] ^{13}C-NMR assignments (Table 2) were made by the analysis of the HMQC, HMBC, and DEPT spectra in conjuction with the comparison with those of mangostanin and caloxanthone D. The structure of mangostenone C (**1**) was therefore concluded to be 1,6-dihydroxy-7-methoxy-8-(3-methylbut-2-enyl)-4′-hydroxy-5′-(1-hydroxy-1-methylethyl)-4′,5′-dihydrofurano(2′,3′:3,2)xanthone. Due to the scarcity of compound **1**, the existing data did not permit the assignment of the absolute stereochemistry at C-11 and C-12 for this compound.

Mangostenone D (**2**) was obtained as a yellow solid. A pseudo molecular ion at *m/z* 397.1646 in the positive-ion HR-FAB-MS established the molecular formula of $C_{23}H_{24}O_6$. Its UV and IR data suggested that **2** also has a xanthone skeleton. In the ^{1}H-NMR spectrum, signals for two aromatic protons (δ 6.38, 6.73), a prenyl group (δ 3.34, 5.27, 1.77, 1.63), and a 2,2-dimethylchroman ring (δ 3.47, 1.87, 1.34) were observed, in addition to three hydroxyl groups (δ 13.79, 9.55, 8.79) (Table 1). The ^{1}H-NMR data of **2** were similar to those of garcinone B (**6**) except that the two doublets at H-16 (δ 8.00, J=10.2 Hz) and H-17 (δ 5.80, J=10.2 Hz) of the dimethylchromene ring in garcinone B[10] were replaced by the two triplet methylenes H-16 and H-17 in **2**. This structural assignment was confirmed by its HMBC spectrum (Fig. 1). ^{13}C-NMR assignments (Table 2) were established by comparison with those of garcinone B (**6**)[10] and mangostenone B.[10] The structure of **2** was therefore assigned to be 1,3,6-trihydroxy-2-(3-methylbut-2-enyl)-6′,6′-dimethyl-4′,5′-dihydropyrano(2′,3′:7,8)xanthone, which is a 16,17-dihydro analogue of garcinone B and designated mangostenone D.

Table 2. IC_{50} Values (μg/ml) for Cytotoxic Activities against KB, BC-1 and NCI-H187 Cancer Cell Lines of Xanthones from *G. mangostana* Fruit

Compound	KB	BC-1	NCI-H187
Mangostenone C (1)	2.8	3.53	3.72
Mangostenone D (2)	9.79	3.88	9.07
Mangostenone E (3)	19.96	17.53	Inactive[a]
Thwaitesixanthone (4)	Inactive[a]	Inactive[a]	Inactive[a]
Demethylcalabaxanthone (5)	10.9	2.85	3.13
Garcinone B (6)	Inactive[a]	Inactive[a]	Inactive[a]
1,6-Dihydroxy-7-methoxy-8-(3-methylbut-2-enyl)6′,6′-dimethylpyrano(2′,3′:3,2)xanthone (7)	3.72	3.02	2.19
β-Mangostin (8)	2.5	2.04	2.88
8-Desoxygartanin (9)	Inactive[a]	Inactive[a]	16.88
Gartanin (10)	15.63	15.54	1.08
Garcinone E (11)	2.67	1.44	3.74
α-Mangostin (12)	2.08	0.92	2.87
Mangostinone (13)	12.79	7.26	17.88
γ-Mangostin (14)	4.69	1.6	2.55
Mangostanol (15)	Inactive[a]	Inactive[a]	1.15
Mangostanin (16)	Inactive[a]	Inactive[a]	8.04
Garcinone D (17)	3.56	2.81	11.04
Garcinone C (18)	7.48	2.18	3.66
11-Hydroxy-1-isomangostin (19)	13.14	18.53	Inactive[a]
Standard ellipticine	1.33	1.46	0.39

a) Inactive at 20 μg/ml.

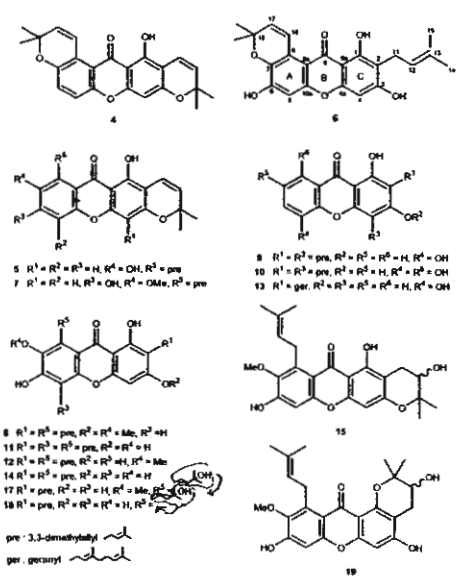

Fig. 2. Chemical Structures of Compounds 2, 4—15 and 17—19

Mangostenone E (3) was obtained as a yellow amorphous solid. The HR-FAB-MS (negative-ion mode) exhibited an $[M-H]^-$ ion at *m/z* 443.1709 compatible with the molecular formula $C_{24}H_{28}O_8$. Its UV and IR data were also indicative of a xanthone derivative. The ^{1}H-NMR spectrum (Table 1) displayed signals for a 1,3,5,6-tetraoxygenated xanthone, which included two aromatic singlets (δ 6.40, 6.84), a methoxyl (δ 3.85), a prenyl group (δ 3.33, 5.27, 1.76, 1.62), and two hydroxyl protons at δ 13.58 (chelated) and 9.86, in addition to a modified prenyl group. Comparison of the ^{1}H- and ^{13}C-NMR data of 3 with those of garcinone D (17)[11] suggested that 3 only differed from compound 17 in the nature of the substituent at the 8-position. In this respect, the presence of two methyls (δ_H 1.29, δ_C 26.1, 25.4), a quaternary oxygenated carbon (δ_C 73.2), an oxygenated methine (δ_H 3.69, δ_C 79.7), and a methylene signal (δ_H 3.59, δ_C 29.5) in the ^{1}H- and ^{13}C-NMR spectra (Table 1) established a 2,3-dihydroxy-3-methylbutyl moiety.[19] In the HMBC spectrum (Fig. 1) the proton resonating at δ 3.59 (H-16) showed correlations with C-7 (δ 145.5), C-8 (δ 137.0), C-8a (δ 112.6), and C-17 (δ 79.7), whereas H-17 (δ 3.69) was correlated with C-19 (δ 26.1) and C-20 (δ 25.4). Strong NOE interactions observed between the methoxyl proton and H-16 and H-17 in the NOESY spectrum (Fig. 1) further supported the placement of the 2,3-dihydroxy-3-methylbutyl group at C-8 in 3. The chemical shifts of C-16, C-17, and C-18 of compound 3 were inconsistent with those reported for cudraxanthone N, a xanthone isolated from *Cudrania tricuspidata*.[19] ^{13}C-NMR assignments (Table 2) were established by comparison with those of garcinone D[10] and cudraxanthone N.[19] On the basis of all spectroscopic evidence, the structure of mangostenone E (3) was established to be 1,3,6-trihydroxy-8-(2,3-dihydroxy-3-methylbutyl)-2-(3-methylbut-2-enyl)-7-methoxyxanthone.

Compounds 4—19 were identified as thwaitesixanthone (4),[12] demethylcalabaxanthone (5),[11] garcinone B (6),[10] compound 7,[11] β-mangostin (8),[10] 8-desoxygartanin (9),[13] gartanin (10),[14] garcinone E (11),[15] the major metabolite α-mangostin (12),[10] mangostinone (13),[10] the second major metabolite γ-mangostin (14),[11] mangostanol (15),[10] mangostanin (16),[11] garcinone D (17),[11] garcinone C (18),[16] and the third major metabolite 11-hydroxy-1-isomangostin (19)[17] by comparison of their ^{1}H- and ^{13}C-NMR, MS and $[\alpha]_D$ data with those reported previously.

Xanthones 1—19 were evaluated *in vitro* for their cytotoxicities against KB, BC-1, and NCI-H187 cells and the IC_{50} values are shown in Table 2. Based on these observations, the following conclusions can be drawn regarding these isolates: 1) For high activity, the xanthones should contain tetraoxygen functions with two C_5 units in rings A and C (as in 8, 11, 12, 14). Among these, α-mangostin (12), the major constituent, exhibited the most potent effects against KB and BC-1 with IC_{50} values of 2.08 and 0.92 μg/ml, respectively. In NCI screening, gartanin (10) demonstrated selective and the most potent activity with the IC_{50} value of 1.08 μg/ml. 2) The activity was generally reduced with the increase in the number of hydroxyl groups in the C_5 side chain (comparison of compounds 12 and 14 with 17, 3, and 18). 3) The weak activity of the third major constituent 19, compared with the highest activity of 12, indicated the crucial role of the hydroxyl group at C-1 in the xanthone nucleus. 4) Cyclization of the C_5 group in either the 1,3,7-trioxygenated xanthone or the 1,3,6,7-tetraoxgenated nucleus resulted in decreased activity (compounds 4 and 6). It should be noted that the pyrano and furano rings bearing the hydroxyl group attached to the xanthone nucleus (as for 1 and 15) appear to enhance cytotoxic activity in NCI screening.

Experimental

General Procedures Melting points were determined with a Griffin melting point apparatus and are uncorrected. Optical rotations were recorded on a Jasco P1010 digital polarimeter. UV spectra were run on a Shimadzu UV-2401 PC spectrophotometer. IR spectra were measured on a Perkin-Elmer FT-IR Spectrum BX spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AVANCE 300 FT-NMR spectrometer, operating at 300 MHz (^{1}H) and 75 MHz (^{13}C). For the spectra taken in $CDCl_3$ and acetone-d_6, residual nondeuterated solvent signals at δ 7.24 and 2.04 and the solvent signals at δ 77.00 and 29.80 were used as references for ^{1}H- and ^{13}C-NMR spectra, respectively. EI-MS and FAB-MS were recorded on a Thermo Finnigan Polaris Q and a Finnigan MAT 90 instrument. Unless otherwise indicated, column chromatography and TLC were carried out using Merck silica gel 60 (finer than 0.063 mm) and precoated silica gel 60 F_{254} plates, respectively. Spots on TLC were visualized under UV light and by spraying with anisaldehyde–H_2SO_4 reagent followed by heating.

Plant Material The young fruit, at 7 weeks of maturity after anthesis, of *G. mangostana* were collected from Ra-ngae district, Narathiwat province, Thailand, in 2000 and a voucher specimen (RU 0038) is deposited at the Faculty of Science, Ramkhamhaeng University, Thailand.

Extraction and Isolation Air-dried and powdered fruit (2.06 kg) of *G. mangostana* was successively extracted with EtOAc and MeOH at 50 °C in a water bath for 48 h each and the solvents were evaporated to yield the EtOAc (295 g) and MeOH (251 g) extracts, respectively. The EtOAc extract, which exhibited stronger cytotoxicity against the KB and BC cell lines, was then investigated extensively. Thus the EtOAc-soluble fraction (42 g) was subjected to quick column chromatography[20] over silica gel using a gradient of hexane–CH_2Cl_2, CH_2Cl_2, CH_2Cl_2–EtOAc, EtOAc, and then EtOAc–MeOH (5% increment of polar solvent for 500 ml of each proportion) and were combined into nine main fractions by TLC examination. Fraction 1 (53 mg) was crystallized with hexane to yield thwaitesixanthone (**4**) (10 mg).[12] Fraction 2 (1.33 g) was chromatographed over silica gel using gradient elution of hexane–CH_2Cl_2 (70 : 30) and CH_2Cl_2 in 5% increments of polar solvent to give fractions 2a—m. Fractions 2c (148 mg), 2d (98 mg), and 2k (102 mg) were individually recrystallized with hexane to produce demethylcalabaxanthone (**5**) (20 mg),[11] garcinone B (**6**) (5 mg),[10] and garcinone E (**11**) (23 mg),[15] respectively. Fraction 2e (326 mg) was sequentially fractionated on a silica gel column (in hexane–CH_2Cl_2, 40 : 60 to 5 : 95) to give compound 7 (52 mg)[11] and β-mangostin (**8**) (5 mg).[10] Fractions 2f—h (189 mg) were further fractionated on a silica gel column (hexane–EtOAc, 95 : 5 to 50 : 50) and then on a Sephadex LH-20 column (in MeOH) to afford 8-desoxygartanin (**9**) (18 mg)[13] and gartanin (**10**) (18 mg).[14] Fractions 3—5 were the major metabolite, α-mangostin (**12**) (6.79 g).[10] Similar purification of fraction 6 (340 mg) on column chromatography (silica gel, CH_2Cl_2–MeOH 99.5 : 0.5 to 95 : 5 in 0.5% stepwise elution) gave mangostinone (**13**) (12 mg).[10] Fraction 7 (3.39 g) was crystallized with $CHCl_3$ to furnish γ-mangostin (**14**) (0.89 g),[11] and the filtrate was further chromatographed over silica gel (eluted with hexane–CH_2Cl_2, CH_2Cl_2, CH_2Cl_2–MeOH, and MeOH in 5% increments of polar solvent for 200 ml of each portion) to provide fractions 7a—j. Mangostanol (**15**) (9 mg),[10] mangostenone C (**1**) (4 mg), mangostenone D (**2**) (6 mg), and mangostanin (**16**) (6 mg),[11] were isolated from repeated individual column chromatography (silica gel) of fractions 7g (561 mg, using a hexane–EtOAc gradient system as eluent), 7h (203 mg, eluted with $CHCl_3$–MeOH, 99 : 1 to 5 : 95), and 7i (204 mg, eluted with CH_2Cl_2–MeOH, 99.5 : 0.5 to 90 : 10), respectively. Similar fractionation of fraction 8 (3.75 g), eluted with a gradient of CH_2Cl_2 and CH_2Cl_2–MeOH (70 : 30), yielded 11 fractions, in which garcinone D (**17**) (90 mg)[11] and garcinone C (**18**) (20 mg)[10] were obtained from subsequent column chromatography (silica gel, eluted with a gradient of CH_2Cl_2 and CH_2Cl_2–MeOH) of fraction 8h (2.29 g). Similarly, purification of fraction 9 (4.20 g) using column chromatography (silica gel, CH_2Cl_2–MeOH 99 : 1 to 30 : 70 in 3% increments of the polar solvent) gave five fractions, mangostenone E (**3**) (9 mg) and the third major isolate, *11-hydroxy-1-isomangostin* (**19**) (235 mg),[12] were isolated from two successive column chromatographies (silica gel, CH_2Cl_2–MeOH 97 : 3 to 95 : 5) of fractions 9b (852 mg) and 9d (1.94 g), respectively. The MeOH extract that exhibited weak cytotoxicity contained a small quantity of a number of xanthones which had been isolated from the EtOAc extract.

Mangostenone C (**1**): Yellow solid; mp 126—127 °C; $[\alpha]_D^{27}$ −28.3° (c=0.12, MeOH); UV λ_{max} (MeOH) nm (log ε): 248 (4.39), 312 (3.97), 347 (3.01); *IR* ($CHCl_3$) cm^{-1}: *3584 (sharp), 3400, 1655, 1576, 1462, 1283, 1168,* 1091; ^{1}H- and ^{13}C-NMR data, see Table 1; EI-MS *m/z*: 442 (M^+), 440, 383, 382, 381, 367, 349, 340, 339 (100), 324, 309, 149; HR-FAB-MS (positive-ion mode) *m/z*: 443.1691 $[M+H]^+$ (Calcd for $C_{24}H_{26}O_8$+H: 443.1705).

Mangostenone D (**2**): Yellow solid; mp 208—210 °C; UV λ_{max} (MeOH) nm (log ε): 244 (4.39), 260 (4.40), 317 (4.23), 362 (3.82); IR (KBr) cm^{-1}: 3510 (sharp), 3421, 1644, 1608, 1586, 1463, 1301, 1286, 1269, 1164, 1079, ^{1}H- and ^{13}C-NMR data: see Table 1; HR-FAB-MS (positive-ion mode) *m/z*: 397.1646 $[M+H]^+$ (Calcd for $C_{23}H_{24}O_6$+H: 397.1651).

Mangostenone E (**3**): Yellow amorphous solid; $[\alpha]_D^{28}$ 0.0° (c=0.13, MeOH); UV λ_{max} (MeOH) nm (log ε): 243 (4.27), 258 (4.15), 321 (3.91), 358 (2.98); IR ($CHCl_3$) cm^{-1}: 3421, 2926, 1647, 1607, 1457, 1281, 1192, 1083 cm^{-1}; ^{1}H- and ^{13}C-NMR data: see Table 1; HR-FAB-MS (negative-ion mode) *m/z*: 443.1709 $[M-H]^-$ (Calcd for $C_{24}H_{28}O_8$−H: 443.1705).

^{13}C-NMR data of the xanthones that had not been published are listed below.

Compound **4**: ^{13}C-NMR (*75 MHz, $CDCl_3$*) δ: *183.9 (C-9), 160.5 (C-3),* 158.0 (C-1), 157.0 (C-4a), 152.0 (C-10a), 149.5 (C-7), 132.6 (C-17), 127.2 (C-12), 124.2 (C-6), 120.8 (C-16), 118.1 (C-8), 117.7 (C-5), 115.5 (C-11), 114.5 (C-8a), 104.3 (C-2), 104.0 (C-9a), 94.2 (C-4), 78.2 (C-13), 75.4 (C-18), 28.3 (C-14, C-15), 27.3 (C-19, C-20).

Compound **9**: ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 181.0 (C-9), 160.9 (C-3), *158.6 (C-1), 152.3 (C-4a), 144.4 (C-10a), 144.2 (C-5),* 136.1 (C-13), 133.5 (C-18), 123.8 (C-7), 122.1 (C-17), 121.1 (C-12), 120.8 (C-8a), 119.7 (C-6), 116.8 (C-8), 109.1 (C-2), 105.4 (C-4), 103.2 (C-9a), 25.8 (C-14), 25.6 (C-19), 22.0 (C-16), 21.6 (C-11), 17.9 (C-15 and C-20).

Compound **10**: ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 184.6 (C-9), 161.6 (C- 3), 158.0 (C-1), 153.7 (C-8), 152.4 (C-4a), 142.8 (C-5), 136.1 (C-13), 135.6 (C-10a), 133.9 (C-18), 122.8 (C-6), 121.8 (C-17), 120.9 (C-12), 109.7 (C-7), 109.4 (C-2), 107.0 (C-8a), 105.7 (C-4), 102.1 (C-9a), 25.8 (C-14), 25.6 (C-19), 21.9 (C-16), 21.5 (C-11), 17.9 (C-15 and C-20).

Cytotoxicity Bioassays The cytotoxicity of compounds **1**—**19** was determined employing the colorimetric method as described by Skehan *et al.*[21] The reference substance, ellipticine, exhibited cytotoxic activity against KB, BC-1, and NCI-H187 cells with IC_{50} values of 1.33, 1.46, and 0.39 μg/ml, respectively.

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Ceanothane- and Lupane-type Triterpenes with Antiplasmodial and Antimycobacterial Activities from *Ziziphus cambodiana*

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One new and eight known ceanothane- and lupane -type triterpenes were isolated from the root bark of *Ziziphus cambodiana* Pierre (Rhamnaceae). Based on spectral analyses, the structure of the new compound was elucidated as 3-*O*-(4-hydroxy-3-methoxybenzoyl)ceanothic acid (3-*O*-vanillylceanothic acid) (1), while the known compounds were identified as lupeol (2), betulinaldehyde (3), betulinic acid (4), 2-*O*-*E*-*p*-coumaroyl alphitolic acid (5), alphitolic acid (6), zizyberanalic acid (7), zizyberenalic acid (8) and ceanothic acid (9). Compounds 1, 5 and 8 exhibited significant *in vitro* antiplasmodial activity against the parasite *Plasmodium falciparum*, with inhibitory concentration (IC_{50}) values of 3.7, 0.9 and 3.0 μg/ml, respectively. Compounds 1 and 3–8 showed antimycobacterial activity against *Mycobacterium tuberculosis* with respective MIC values of 25, 25, 25, 12.5, 50, 50 and 100 μg/ml.

Key words *Ziziphus cambodiana*; Rhamnaceae; triterpene; antimycobacterial activity; antiplasmodial activity

The Rhamnaceous *Ziziphus* is well-known for its content of triterpenes (betulinic and alphitolic acids),[1] saponins (jujubisides and joazeirosides)[2, 3] and cyclopeptide alkaloids (lotosines).[4] Some *Ziziphus* plants have been found to possess biological activities, for example sedative,[5] hypoglycemic,[6] antibacterial and antifungal activities.[7] *Ziziphus cambodiana* Pierre is a thorny Rhamnaceous scandent widely distributed in the north-east of Thailand and used traditionally for its antiinfectious abilities.[8] No phytochemical study of this plant has been reported so far. In our search for biologically active substances of new structural types from Thai natural resources,[9–12] we investigated the chemical constituents and biological activities of this plant species and have found that the ethyl acetate extract of the root bark of *Z. cambodiana* showed pronounced *in vitro* antimalarial potential against *Plasmodium falciparum*. In this paper, we report the isolation and characterization of a new tritrpene ester (**1**), together with five known lupane constituents: lupeol (**2**),[13] betulinaldehyde (**3**),[14] the major metabolite betulinic acid (**4**),[13] 2-*O*-*E*-*p*-coumaroylalphitolic acid (**5**),[1] and alphitolic acid (**6**),[1] and three ceanothane triperpenes: zizyberanalic acid (**7**),[15] zizyberenalic acid (**8**)[15] and ceanothic acid (**9**),[16] as the antiplasmodial and antituberculosis constituents of the root bark of this plant species. This is the first report of *in vitro* antiplasmodial and antimycobacterial activities from the ceanothane-type triterpenes.

Compound **1** was obtained as a colorless solid, mp 183–185 °C. Negative high-resolution fast atom bombardment mass spectrometry (HR-FAB-MS) established a pseudomolecular ion at *m/z* 635.3592, compatible with a molecular formula of $C_{38}H_{52}O_8$. This compound exhibited IR absorption bands for hydroxyl (3430 cm^{-1}), conjugated carbonyl ester (1699 cm^{-1}) and aromatic (1602 and 1512 cm^{-1})

functionalities. The ^{1}H-NMR spectrum (Table 1) revealed an isopropenyl group (δ_H 4.82, 4.62, and 1.63), and five additional singlet methyls (δ_H 1.53, 1.23, 1.11, 1.10 and 1.02), as well as two singlet methine protons at δ_H 5.94 and 3.15 which coupled to each other, as suggested by the correlated spectroscopy (COSY) spectrum. In the ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra, 38 carbon signals were observed, including six methyls, one methoxyl, nine methylenes, ten methines, and nine quaternary carbons, as well as one ester carbonyl and two carboxylic acids carbon signals (Table 1). The presence of a vanillyl moiety in the molecule was supported by aromatic protons at δ_H 7.86 (1H, br s), 7.28 (1H, d, J=8.2 Hz) and 7.90 (1H, br d, J=8.2 Hz) and one aromatic methoxyl group at δ_H 3.75 (3H, s). A nuclear Overhauser enhancement (NOE) displayed between H-2′ (δ_H 7.86) and the methoxyl group in the nuclear Overhauser enhancement spectroscopy (NOESY) spectrum, supporting the placement of the methoxyl group at the meta position in the vanillyl unit (Fig.1). The NMR spectral data of **1** are very similar to those of ceanothic acid (**9**), except for the presence of a vanillyl ester moiety in **1**. The ester substituent was placed at C-3 as a result of downfield shifts observed for H-3 and C-3 in the ^{1}H- and ^{13}C-NMR spectra, respectively, as well as from correlations exhibited between H-3 (δ_H 5.94) and C-1 (δ_C 64.3), C-2 (δ_C 176.8) and C-7′ (δ_C 166.3) of the vanillyl group in the heteronuclear multiple bond correlation (HMBC) spectrum. The relative configuration of H-1 and H-3 for compound **1** was further suggested by a NOESY experiment (Fig. 1), wherein NOE enhancements were displayed between H-3 and H-23, H-1 and H-24 and H-25, confirming the H_{β}-1 and H_{α}-3 orientation of the ceanothic acid nucleus. ^{13}C-NMR assignments of **1** were made by one-dimensional (1D-) and 2D-NMR spectral data analysis, and by comparison with those of ceanothic acid[16] and 3-*O*-protocatechuoylceanothic acid.[17] Compound **1**, therefore, was elucidated as 3-*O*-

(4-hydroxy-3-methoxybenzoyl)ceanothic acid or 3-*O*-vanillylceanothic acid. To our knowledge, the triterpene ester **1** is the third novel naturally occurring aromatic acid ester of the ceanothane-type triterpene, the previous ones being 2-*O*-*E*-*p*-coumaroylceanothanolic acid[15] and 3-*O*-protocatechuoylceanothic acid.[17]

The antiplasmodial and antituberculosis activities of the isolates **1-9** against *P. falciparum* and *M. Tuberculosis* were tested, and the 50% inhibitory concentration (IC_{50}), and minimum inhibitory concentration (MIC) values were determined (Table 2). Weak antiplasmodial activities for compounds **2**, **4** and **6**[18, 19] and antimycobacterial activities for compound **2**[20] were previously recorded, however they were in the inactive range of our test. The antituberculosis activity of **4** was similar to the reported value.[20] A comparison of compounds **5** with **6** and **1** with **9** indicated that the *p*-coumarate moiety in **5** and the vanillyl group of compound **1** were crucial for high antiplasmodial and antituberculosis potentials. Introduction of a double bond in ring A of the ceanothane-type triterpene **8** (IC_{50} 3.0 μg/ml) highly increased the inhibitory activity in the antiplasmodial assay as compared to compound **7**.

Experimental

General Experimental Procedures Melting points were determined using a Griffin melting point apparatus. Optical rotations were determined on a Jasco P1010 digital polarimeter. UV spectra were obtained on a Shimadzu UV-2401 PC spectrophotometer. IR spectra were measured on a Perkin Elmer FT-IR Spectrum BX spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AVANCE 300 FT-NMR spectrometer operating at 300 MHz (^{1}H) and 75 MHz (^{13}C). For the spectra taken in pyridine-d_5, the residual nondeuterated solvent signals at δ_H 8.70 and the solvent signals at δ_C 149.9 were used as references for ^{1}H- and ^{13}C-NMR spectra,

respectively. Electron impact (EI) and FAB mass spectra were run on Thermo Finnigan Polaris Q and Finnigan MAT 90 instruments. Unless indicated otherwise, column chromatography and TLC were carried out using Merck silica gel 60 (< 0.063 mm) and precoated silica gel 60 F_{254} plates, respectively. Plates of silica gel PF_{254}, thickness 1.25 mm, were used for preparative TLC. Spots on TLC were visualized under UV light and by spraying with anisaldehyde-H_2SO_4, followed by heating.

Plant Material The dried root bark of *Z. cambodiana* was collected from Chamni District, Burirum Province, Thailand in March, 2001. A voucher specimen (Wicharn Wisetsri 001) is deposited at the CMU Herbarium, Faculty of Science, Chiang Mai University, Thailand.

Extraction and Separation Pulverized, dry root bark (4.85 kg) of *Z. cambodiana* was defatted with hexane and then extracted successively with ethyl acetate (EtOAc) and methanol (MeOH) at 50 °C for 50 h, and the solvents were evaporated to yield EtOAc (60.4 g) and MeOH (629.3 g) extracts, respectively. The EtOAc extract exhibited antiplasmodial and antimycobacterial activities, whereas the MeOH extract was found to be inactive. Thus, the EtOAc soluble fraction was investigated extensively through serial fractionations by quick column chromatography,[21] and eluted with a gradient of hexane, hexane-$CHCl_3$, $CHCl_3$, $CHCl_3$–EtOAc, EtOAc, EtOAc–MeOH, MeOH (5% increment of polar solvent, each 300 ml) to provide six major fractions (Fr. 1–6). Recrystallization of fraction 1 (0.8 g) with hexane and fraction 3 (9.9 g) with CH_2Cl_2, gave **2** (15 mg) and **4** (2.6 g), respectively. The filtrate from fraction 3 (6.9 g) was further subjected to silica gel column chromatography employing solvent gradient hexane-CH_2Cl_2, and ten fractions (fr. 3a to 3j) were collected. Fraction 3d (327 mg) was further separated with silica gel chromatography, and eluted with hexane–CH_2Cl_2 of increasing polarity to yield 11