Isolation of Pyropheophorbide a from the Leaves of *Atalantia monophylla* (ROXB.) CORR. (Rutaceae) as a Possible Antiviral Active Principle against Herpes Simplex Virus Type 2

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Antiviral activity-guided isolation studies on the leaves of *Atalantia monophylla* (ROXB.) CORR. (Rutaceae) led to the identification of pyropheophorbide a (1), a simple chlorin derivative, from the chloroform extract (fr. B) as a possible antiviral active principle against herpes simplex virus type 2 (HSV-2). Pyropheophorbide a methyl ester (2) was also isolated from the hexane extract (fr. A).

Key words pyropheophorbide a; pyropheophorbide a methyl ester; isolation; antiviral activity; *Atalantia monophylla*; Rutaceae

Atalantia monophylla (ROXB.) CORR. (Rutaceae) has been used as a folk medicine for several purposes such as the treatment of dysentery,[1] chronic rheumatism[2] and paralysis[2] in India. As part of the continuous studies[3] on exploring antiviral active lead compounds from natural sources, we focused on the leaves of this plant (called as "Makadi" in India[1]). The biological activity-guided separation studies against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) using acyclovir (ACV), a drug for clinical use, as a standard resulted in the isolation of chlorin derivatives. In this paper we describe the identification of pyropheophorbide a (**1**) as a possible antiviral active principle against HSV-2 and the isolation of the corresponding methyl ester (**2**).

Results and Discussion

The dried and powdered leaves of this plant were extracted by Soxhlet apparatus using hexane (fr. A), chloroform (fr. B) and methanol (fr. C). When each extract was tested for antiviral activities against HSV-1 and HSV-2, moderate activity was found only in fr. B (see Table 1). Fraction B was roughly separated into five fractions (fr. 1 to fr. 5) by column chromatography (CC) using a gradient system of chloroform and chloroform–methanol (Chart 1). Although all of the fractions showed no antiviral activity against HSV-1, the antiviral activity against HSV-2 remained in the more polar fr. 3 and fr. 4 among the five (see Table 1). A characteristic reddish-colored spot detected by UV (365 nm) was observed in the TLC of fr. 4. Further purification of fr. 4 by flash chromatography (FC) and preparative thin layer chromatography (PLC)[4] led to the isolation of a green-colored amorphous mass.

The isolated product showed hydroxyl and carbonyl absorption bands at 3394 and 1734 cm^{-1}, respectively in the IR spectrum. The molecular formula of $C_{33}H_{34}N_4O_3$ was deduced by the appearance of a peak at *m/z*: 534.2651 (Calcd for $C_{33}H_{34}N_4O_3$: 534.2613) in the high resolution fast atom bombardment MS (HR-FAB-MS). The characteristic absorption maxima (410, 509, 538, 608, 667 nm) at a visible region in the UV spectrum suggested

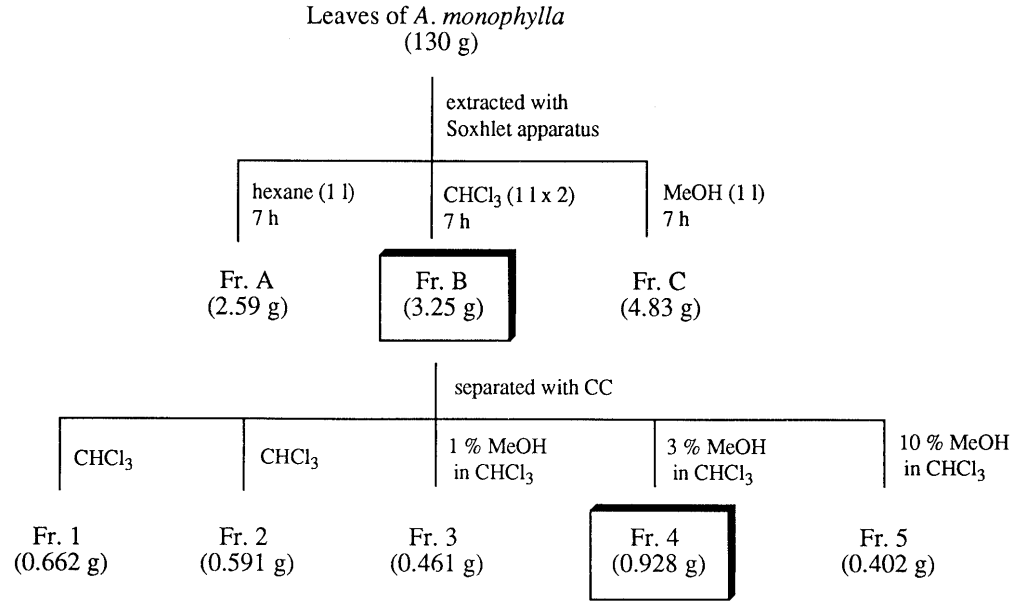

Chart 1. A Flow Chart for Antiviral Activity-Guided Separation of the Leaves of *A. monophylla*

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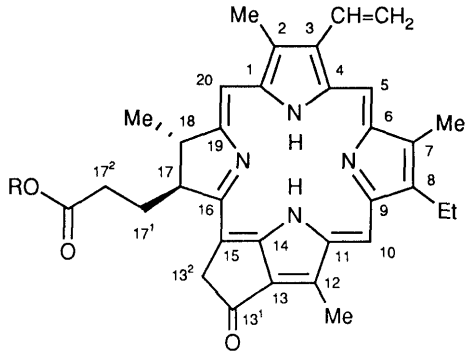

Fig. 1

Table 1. Antiviral Activities of the Crude Fractions, Pyropheophorbide a (**1**) and ACV[a]

Substrates and fractions	EC_{50} (μg/ml)	
	HSV-1	HSV-2
Fr. A	>100	>100
Fr. B	31	24
Fr. C	>100	76
Fr. 1	>100	>100
Fr. 2	>100	>100
Fr. 3	>100	25
Fr. 4	>100	18
Fr. 5	>100	67
1	>100	57
ACV	0.2	0.2—0.3

a) Plaque reduction assays for HSV-1 (strain KOS) and HSV-2 (strain 186) were performed with Vero cell monolayers by the reported procedure[9] with a slight modification. Inhibition of plaque development for both viruses was evaluated on monolayers after 1 to 2 d incubation at 37 °C. EC_{50} values were determined from the drug concentration which conferred 50% plaque reduction compared to virus controls.

that this green-colored compound could be a chlorin derivative.[5] The ^{1}H-NMR spectrum gave relatively well-separated signals, in which three 3H singlets due to methoxyl and/or deshielded C-methyl groups appeared at δ 3.20, 3.38 and 3.61. Deshielded C-ethyl [δ 1.68 (3H, t, J=7.5 Hz), 3.65 (2H, q, J=7.5 Hz)] and a vinyl [δ 6.25 (1H, dd, J=11.7, 1.5 Hz), 6.16 (1H, dd, J=17.5, 1.5 Hz), 7.96 (1H, dd, J=17.5, 11.7 Hz)] groups were observed as additional functions. Furthermore, the presence of a γ, δ-disubstituted hexanoyl unit of $MeC_\delta HC_\gamma HC_\beta H_2C_\alpha H_2$-CO [$\delta$ 1.81 (3H, d, J=7.5 Hz), 2.18 (1H, m), 2.30 (1H, m), 2.65 (2H, m), 4.28 (1H, dif d, J=8.8 Hz), 4.45 (1H, dq, J=7.5, 2.5 Hz)] was indicated. A small coupling constant of J=2.5 Hz between γ-and δ-methine protons of this sequence showed that their dihedral angle should be near 90°. Combining these partial structures based on the spectral data allowed us to deduce the isolated product as pyropheophorbide a[6] (**1**). The ^{13}C- and two-dimensional (2D) NMR spectra of **1** reasonably supported the above deduction.

During independent separation work on fr. A, another less polar green-colored amorphous mass was isolated. Detailed examination of its spectral data compared to those of **1**, which led to the deduction of it as pyropheophorbide a methyl ester (**2**), which was given by methylation of **1** with dimethyl sulfate in dimethylformamide in the presence of potassium carbonate.

Pyropheophorbide a (**1**) and its methyl ester (**2**) were isolated as optically active forms (see Experimental). The absolute configurations at both the 7 and 8 positions of these products should be safely assigned to be *S* because all of the natural chlorin derivatives had been determined to be *S* at those positions.

Tests of pyropheophorbide a (**1**) for antiviral activities against HSV-1 and HSV-2 showed modest activity against HSV-2 (EC_{50}=57 μg/ml). Recently, new chlorophyll a related compounds have been isolated as antioxidants from marine organisms.[6] This is the first identification of a chlorin derivative as a possible antiviral active principle. However, loss of the activity was observed during purification. Therefore, a real active principle may be contained as minor components.

Several chemical constituents, such as acridone alkaloids, coumarins, limonoids and sterols, have been isolated mainly from the root of this plant.[2,7,8] On the other hand, only neutral components like lipid, triterpenes, sterols and essential oils have been isolated from the leaves.[8] Further studies on the isolation of a promising active principle from this plant are in progress at present.

Experimental

IR and UV spectra were recorded on JASCO FT/IR-300E and Hitachi U-3400 spectrophotometers, respectively. CD spectra were recorded on a JASCO J-500 spectropolarimeter. ^{1}H-NMR spectra were recorded in $CDCl_3$ solution with a JEOL JNM GSX-500α (500 MHz) spectrometer with tetramethylsilane as an internal reference. HR-FAB-MS was recorded on a JEOL JMS-HX 110A spectrometer with a direct inlet system. For CC and FC, Silica gel 60 (70—230 mesh ASTM; Merck) and Silica gel 60 (230—400 mesh ASTM; Merck) were used, while for TLC and PLC, Silica gel G_{F254} (Merck) were used.

Extraction of the Leaves of *A. monophylla* The powdered leaves (130 g) of *A. monophylla* collected in Bangkok, Thailand in 1995, were extracted by Soxhet apparatus as shown in Chart 1 to give three fractions (fr. A, B, C).

Treatment of Fr. B Fraction B was roughly separated by CC into five fractions (fr. 1 to fr. 5), as shown in Chart 1.

Pyropheophorbide a (1) Purification of fr. 4 by CC and FC using a gradient of $CHCl_3$ and $CHCl_3$–MeOH afforded a green-brownish amorphous mass (0.147 g, 0.113%), a part of which was further purified by PLC (hexane : ethyl acetate = 1 : 1) as the only isolable product. IR ν_{max} (KBr) cm^{-1}: 3394 (NH), 1734 (CO). UV λ_{max} (MeOH) nm: 410, 509, 538, 608, 667. ^{1}H-NMR δ: 1.68 (3H, t, J=7.5 Hz, $CH_2C\underline{H}_3$), 1.81 (3H, d, J=7.5 Hz, 18-Me), 2.18, 2.30 (each 1H, m, 17^1-H_2), 2.65 (2H, m, 17^2-H_2), 3.20 (3H, s, CMe), 3.38 (3H, s, CMe), 3.61 (3H, s, CMe), 3.65 (2H, q, J=7.5 Hz, $C\underline{H}_2CH_3$), 4.28 (1H, d, J=8.8 Hz, 17-H), 4.45 (1H, dq, J=7.5, 2.5 Hz, 18-H), 5.15, 5.24 (each 1H, d, J=20.0 Hz, 13^2-H_2), 6.16 (1H, dd, J=11.7, 1.5 Hz, CH=C$\underline{H}$H), 6.25 (1H, dd, J=17.5, 1.5 Hz, CH=CH$\underline{H}$), 7.96 (1H, dd, J=17.5, 11.7 Hz, C$\underline{H}$=CH_2), 8.52, 9.32, 9.43 (each 1H, s, olefinic H). ^{13}C-NMR δ: 11.21 (CH_3), 12.02 (CH_3), 12.08 (CH_3), 17.40 ($CH_2\underline{C}H_3$), 19.42 ($\underline{C}H_2CH_3$), 23.13 (CH_3), 29.64 (CH_2), 30.61 (CH_2), 47.96 (CH_2), 49.95 (CH), 50.86 (CH), 51.50 (CH), 93.01 (CH), 97.13 (CH), 104.09 (CH), 105.95 (C), 122.52 (CH_2), 128.30 (C), 130.32 (C), 131.59 (C), 135.82 (C), 136.05 (C), 136.24 (C), 137.79 (C), 141.58 (C), 144.98 (C), 149.02 (C), 150.74 (C), 155.27 (C), 160.21 (C), 176.98 (CO), 196.51 (CO). CD (c=3×10^{-4}, $CHCl_3$) $[\theta]^{24}$ (nm): 0 (690), −1267 (665), 0 (585—552), 567 (535), 0 (518—450), +1933 (412), 0 (384), −600 (365), 0 (338), +500 (323), 0 (312), −300 (298). HR-FAB-MS m/z: 534.2651 (Calcd for $C_{33}H_{34}N_4O_3$: 534.2613).

Methylation of 1 A mixture of **1** (0.0010 g), Me_2SO_4 (0.1 ml) and K_2CO_3 (0.0052 g) in dimethyl formamide (0.5 ml) was stirred at room temperature for 2 h. Work-up of the reaction mixture gave a methylated product (**2**), which was identical with the natural pyropheophorbide a

methyl ester described below.

Separation of Fr. A: Pyropheophorbide a Methyl Ester (2) Purification of fr. A by CC (hexane : AcOEt = 2 : 1) afforded a green-brownish amorphous mass (0.0152 g, 0.014%), a part of which was further purified by PLC (hexane : $CHCl_3$ = 1 : 5). UV λ_{max} (MeOH) nm: 409, 507, 538, 608, 665. ^{1}H-NMR δ: 1.62 (3H, t, J = 7.5 Hz, $C\underline{H}_2CH_3$), 1.74 (3H, d, J = 7.3 Hz, 18-Me), 2.23, 2.48 (each 1H, m, 17^1-H_2), 2.67 (2H, m, 17^2-H_2), 3.17 (3H, s, CMe), 3.33 (3H, s, CMe), 3.56 (3H, s, OMe), 3.60 (3H, s, CMe), 3.65 (2H, q, J = 7.5 Hz, $C\underline{H}_2CH_3$), 4.22 (1H, d, J = 8.8 Hz, 17-H), 4.43 (1H, dq, J = 7.3, 2.4 Hz, 18-H), 5.03, 5.19 (each 1H, d, J = 20.0 Hz, 13^2-H_2), 6.09 (1H, dd, J = 11.7, 1.5 Hz, CH = C$\underline{H}$H), 6.21 (1H, dd, J = 18.0, 1.5 Hz, CH = C$\underline{H}$H), 7.95 (1H, dd, J = 18.0, 11.7 Hz, C$\underline{H}$ = CH_2), 8.48, 9.32, 9.42 (each 1H, s, olefinic H). ^{13}C-NMR δ: 11.24 (CH_3), 12.05 (CH_3), 12.08 (CH_3), 17.42 ($CH_2\underline{C}H_3$), 19.47 ($\underline{C}H_2CH_3$), 23.12 (CH_3), 29.87 (CH_2), 30.91 (CH_2), 48.02 (CH_2), 49.98 (CH), 51.66 (CH), 51.67 (OCH_3), 92.97 (CH), 97.16 (CH), 104.09 (CH), 105.95 (C), 122.55 (CH_2), 128.28 (C), 129.20 (C), 130.40 (C), 131.53 (C), 135.82 (C), 136.10 (C), 137.67 (C), 145.04 (C), 148.90 (C), 150.86 (C), 155.38 (C), 160.27 (C), 171.40 (C), 173.51 (CO), 196.26 (CO). CD ($c = 1.82 \times 10^{-4}$, $CHCl_3$) $[\theta]^{24}$ (nm): 0 (693), −2308 (663), 0 (570—550), 989 (535), 0 (513—450), +3407 (412), 0 (383), −989 (365), 0 (339), +989 (323), 0 (308), −440 (296). HR-FAB-MS m/z: 548.2777 (Calcd for $C_{34}H_{36}N_4O_3$: 548.2796).

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