Antimycobacterial and Antiplasmodial Unsaturated Carboxylic Acid from the Twigs of *Scleropyrum wallichianum*

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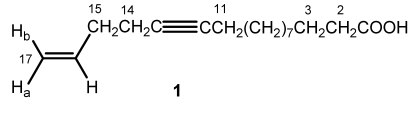
From the twigs of *Scleropyrum wallichianum*, a new unsaturated carboxylic acid, scleropyric acid (1), two new esters, β-sitosteryl-3-*O*-scleropyrate (2) and stigmasteryl-3-*O*-scleropyrate (3), and two well-known sterols, β-sitosterol (4) and stigmasterol (5), were isolated and characterized using spectroscopic methods. Compound 1 exhibited antimycobacterial activity with an MIC value of 25 μg/ml and showed antiplasmodial activity with an IC_{50} value of 7.2 μg/ml. Compounds 2 and 3 were inactive in both assays.

Key words *Scleropyrum wallichianum*; scleropyric acid; β-sitosteryl-3-*O*-scleropyrate; stigmasteryl-3-*O*-scleropyrate; antimycobacterial activity; antiplasmodial activity

Scleropyrum wallichianum (WIGHT & ARN.) ARN. (synonym *Scleropyrum maingayi* HOOK. f.) is a small tree in the Santalaceae family. Previous phytochemical investigation of the seed oil of this plant species revealed the presence of two acetylenic acids, octadec-9-ynoic acid and 17-octadecen-9-ynoic acid, and 11 other fatty acids, decanoic, lauric, palmitic, stearic, arachidic, behenic, hexadecanoic, oleic, eicosenoic, erucic, and linoleic acids.[1)] As part of our work on the bioactive constituents of medicinal plants for the remedy of tropical diseases, we discovered that the hexane extract of this plant species exhibited antimycobacterial and antiplasmodial activities. The present communication deals with the isolation and structure elucidation of a new unsaturated carboxylic acid, scleropyric acid (**1**), and two new carboxylic esters, β-sitosteryl-3-*O*-scleropyrate (**2**) and stigmasteryl-3-*O*-scleropyrate (**3**). The well-known sterols β-sitosterol (**4**) and stigmasterol (**5**) were also isolated and identified. The known sterols **4** and **5** were identified by direct comparison (TLC, ^{1}H-NMR, and EI mass spectra) with authentic **4** and **5**, respectively.

Scleropyric acid (**1**) was obtained as colorless sticky solid. The HR-FAB mass spectrum (negative-ion mode) established a pseudomolecular ion $[M-H]^-$ at *m/z* 263.2013, compatible with the molecular formula of $C_{17}H_{28}O_2$. The IR absorption bands at 3400—3200 and 1708 cm^{-1} indicated the presence of a carboxylic group, whereas that at 1641 cm^{-1} revealed the presence of an olefinic functional group. The ^{13}C-NMR spectrum of **1** revealed 12 saturated methylene carbons, one carbonyl carbon, two acetylenic carbons, and two olefinic carbons, the latter of which corresponded to one methylene and one methine carbons. The presence of a carboxylic group was confirmed by the carbonyl carbon resonance at δ 179.7 in the ^{13}C-NMR spectrum. The two-proton triplet (J=7.4 Hz) at δ 2.32 was assigned to H-2 as evident from the HMBC correlations with C-1 and C-3. The two-proton quintet (J=7.4 Hz) at δ 1.62 was assigned to H-3 which showed a COSY cross peak with H-2 and exhibited HMBC correlations with C-1, C-2, and C-4. The presence of a terminal olefinic moiety was evident from the methylene signal of H-17a and H-17b at δ 4.91 and 4.96, which coupled to H-16 (J=10.2, 17.0 Hz), then coupled to each other and further long-range coupled to H_2-15 to give apparent J values of 0.7 and 1.2 Hz, respectively. The ddt signal (J=17.0, 10.2, 6.8 Hz) of H-16 appeared at δ 5.78 in the ^{1}H-NMR spectrum. The ^{13}C-NMR signals of the olefinic C-16 and C-17 appeared at δ 139.1 and 114.1. The presence of an acetylenic function was evident from the presence of two ^{13}C-NMR signals at δ 80.0 and 80.3. Although the acetylenic absorption band was absent in the IR spectrum, this observation is normal for a disubstituted alkyne.[2)] The four-proton signals at δ 2.11 were assigned to the two methylene groups attached to the acetylenic group (*i.e.*, H_2-11 and H_2-14) which showed HMBC connectivities with C-12 and C-13. The allylic methylene proton and carbon resonances at the 15-position were at δ 2.02 and δ 33.7 in the ^{1}H- and ^{13}C-NMR spectra, respectively. This proton signal exhibited COSY cross peaks with H-16 and H_2-17 and showed HMBC correlations with C-16 and C-17.

The EI fragment peaks at *m/z* 149, 135, 121, 107, 93, 79, and 55 corresponded, respectively, to C–C bond fissions between C-6 and C-7, C-7 and C-8, C-8 and C-9, C-9 and C-10, C-10 and C-11, C-11 and C-12, and C-13 and C-14, giving rise to fragment ions bearing the unsaturated portion. The



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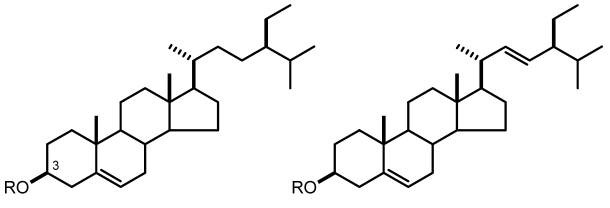

2 : R = $OC(CH_2)_{10}C\equiv C(CH_2)_2CH=CH_2$ (1' … 17') **3** : R = $OC(CH_2)_{10}C\equiv C(CH_2)_2CH=CH_2$

4 : R = H **5** : R = H

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base peak at *m*/*z* 79 confirmed that only two methylene group were present between the olefinic and acetylenic functions. The peak at *m*/*z* 185 which resulted from the cleavage between the C-11 and C-12 bond to give the fragment ion carrying the carboxylic function also confirmed the number of the methylene groups between the two unsaturated functional groups in **1**. The structure of the new unsaturated carboxylic acid was thus concluded to be **1** and designated scleropyric acid.

The ^{1}H-NMR features of compound **2** were similar to those of a mixture of compound **1** and β-sitosterol (**4**). The ES mass spectrum showed a pseudomolecular ion at *m*/*z* 683, corresponding to the $[M+Na]^+$ ion of the β-sitosteryl ester of scleropyric acid. The IR absorption band at 1736 cm^{-1} is indicative of the aliphatic ester group. A 1.09-ppm downfield shift of the H-3 signal, shifting from δ 3.50 in the spectrum of **4** to δ 4.59 in that of **2**, indicated the ester linkage between compounds **1** and **4**. Alkaline hydrolysis of **2** furnished **1** and **4**. The identities of **1** and **4** were confirmed by TLC comparisons with the authentic compounds and, in the case of compound **1**, EI mass spectral comparison. The ester **3** exhibited ^{1}H-NMR spectral features similar to those of compound **2**. The significant differences were the presence of two double doublets of the side-chain olefinic protons at δ 4.99 and 5.13. Alkaline hydrolysis of **3** gave two products identified as the acid **1** and the sterol **5** by the same analogy to those of compounds **1** and **4** mentioned above. Compounds **2** and **3** were thus concluded to be β-sitosteryl-3-*O*-scleropyrate and stigmasteryl-3-*O*-scleropyrate, respectively.

Compound **1** showed moderate antimycobacterial activity with an MIC value of 25 μg/ml and exhibited weak antiplasmodial activity with the IC_{50} value of 7.2 μg/ml. However, compounds **2** and **3** were inactive in both assays.

Experimental

General Procedures IR spectra were recorded on a Perkin-Elmer FT-IR Spectrum BX spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance 400 FT-NMR spectrometer, operating at 400 MHz (^{1}H) and 100 MHz (^{13}C). For spectra taken in $CDCl_3$, the residual nondeuterated solvent signals at δ 7.24 and 77.00 were used as references for ^{1}H- and ^{13}C-NMR spectra, respectively. EI-MS and ES-MS were measured with a Finnigan Polaris Q and a Bruker Esquire-LC mass spectrometer. High-resolution FAB-MS were measured with a Finnigan MAT 90 instrument. Unless indicated otherwise, 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 twigs of *S. wallichianum* were collected from Phuphan district, Sakon Nakhon province, Thailand, in July 2001. A voucher specimen (Apichart Suksamrarn, No. 002) is deposited at the Department of Biology, Faculty of Science, Khon Kaen University.

Extraction and Isolation The pulverized, dry twigs (1.06 kg) were extracted successively with *n*-hexane, $CHCl_3$, and MeOH in a Soxhlet extraction apparatus to yield the hexane (7.12 g), $CHCl_3$ (3.50 g), and MeOH (12.42 g) extracts, respectively. The hexane extract (7.00 g) was chromatographed (Merck silica gel, 0.063—0.200 mm), using a gradient of hexane, hexane–$CHCl_3$, $CHCl_3$ and $CHCl_3$–MeOH (5% increment in the polar solvent for 250 ml of each proportion) to give eight main fractions. Fraction 3 (502 mg) was chromatographed using a gradient of hexane–$CHCl_3$ and $CHCl_3$ in increasing proportions of the polar solvent to give seven subfractions. Subfraction 2 was subsequently chromatographed and fractions eluted by hexane–$CHCl_3$ (99.5 : 0.5) were rechromatographed in a similar manner to yield a mixture of **2** and **3** (5 mg) which showed a homogeneous spot on TLC. Subfraction 5 was similarly chromatographed and fractions eluted by hexane–$CHCl_3$ (99 : 1) yielded **4** (2 mg), a mixture of **4** and **5** (7 mg), and **5** (2 mg). Fraction 6 (112 mg) was chromatographed using hexane–$CHCl_3$ (from 99 : 1 to 96 : 4) and the fraction eluted by hexane–$CHCl_3$ (97 : 3) was rechromatographed twice to afford **1** (14 mg). Another lot (7 mg) of a mixture of compounds **2** and **3** was obtained from the second lot of the pulverized, dry twigs (1.2 kg) by similar treatments of the hexane extract. The two lots of the compound **2** and **3** mixture were combined and subjected to column chromatography using hexane–EtOAc (8 : 0.05) as an isocratic eluting solvent. The purity of compounds **2** and **3** in each eluate was determined by ^{1}H-NMR investigation. After four repeated column chromatographies, the pure ester **2** (3.0 mg) was obtained. The ester **3** (3.5 mg) was similarly obtained after four repeated column chromatographies.

Scleropyric Acid (**1**): Colorless sticky solid, IR (neat) cm^{-1}: 3400—3200 (br), 3072, 2928, 2862, 1708, 1641, 1458, 1435, 1418, 1282, 1223, 1129, 1075, 991, 909. EI-MS *m*/*z* (rel. int.): 264 $[M]^+$ (4), 185 (6), 149 (32), 135 (44), 121 (39), 107 (44), 93 (65), 79 (100), 55 (33). HR-FAB-MS (negative-ion mode) *m*/*z*: 263.2013 $[M-H]^-$ (Calcd for $C_{17}H_{28}O_2$–H: 263.2011). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.20—1.35 (5×2H, m, 5×CH_2), 1.38—1.45 (2×2H, m, 2×CH_2), 1.62 (2H, quintet, J=7.4 Hz, H_2-3), 2.02 (2H, m, H_2-15), 2.11 (2×2H, m, H_2-11 and H_2-14), 2.32 (2H, t, J=7.4 Hz, H_2-2), 4.91 (1H, apparent dd, J=10.2, 0.7 Hz, H-17a), 4.96 (1H, apparent dd, J=17.0, 1.2 Hz, H-17b), 5.78 (1H, ddt, J=17.0, 10.2, 6.8 Hz, H-16). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 18.70, 18.74 (C-11, C-14), 24.6 (C-3), 28.4, 28.60, 28.64, 28.69, 28.84, 28.89, 29.1 (C-4 to C-10), 33.7 (C-15), 33.9 (C-2), 80.0, 80.3 (C-12, C-13), 114.1 (C-17), 139.1 (C-16), 179.7 (C-1). HMBC correlations (100 MHz, $CDCl_3$): H-2 (C-1, C-3), H-3 (C-1, C-2, C-4), H-11 and H-14 (C-12, C-13), H-15 (C-16, C-17), H-16 (C-15), H-17 (C-15, C-16).

β-Sitosteryl-3-*O*-scleropyrate (**2**): Amorphous, IR (KBr) cm^{-1}: 2935, 2855, 1736, 1641, 1466, 1451, 1383, 1272, 1220, 1180, 1028, 920. ES-MS (positive-ion mode) *m*/*z*: 683 $[M+Na]^+$. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.65 (3H, s, 18-Me), 0.79 (3H, t, J=7.0 Hz, 29-Me), 0.80—0.82 (2×3H, partially overlapping signals, 26-Me, 27-Me), 0.90 (3H, d, J=6.4 Hz, 21-Me), 0.99 (3H, s, 19-Me), 1.20—1.50 (multiplet signals of CH, CH_2), 1.60 (2H, quintet, J=7.4 Hz, H_2-3′), 2.02 (2H, m, H_2-15′), 2.11 (2×2H, m, H_2-11′, H_2-14′), 2.25 (2H, t, J=7.5 Hz, H_2-2′), 4.59 (1H, m, H-3), 4.91 (1H, apparent dd, J=10.2, *ca.* 0.5 Hz, H-17′a), 4.97 (1H, apparent dd, J=17.1, 1.7 Hz, H-17′b), 5.35 (1H, br d, J=*ca.* 4.0 Hz, H-6), 5.78 (1H, ddt, J=17.1, 10.2, 6.8 Hz, H-16′).

Stigmasteryl-3-*O*-scleropyrate (**3**): Amorphous, IR (KBr) cm^{-1}: 2933, 2854, 1736, 1640, 1465, 1451, 1383, 1272, 1220, 1180, 1027, 919. ES-MS (positive-ion mode) *m*/*z*: 681 $[M+Na]^+$. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.67 (3H, s, 18-Me), 0.78 (3H, t, J=6.9 Hz,, 29-Me), 0.78—0.80 (2×3H, partially overlapping signals, 26-Me, 27-Me), 0.82 (3H, d, J=6.2 Hz, 21-Me), 0.99 (3H, s, 19-Me), 1.20—1.50 (multiplet signals of CH, CH_2), 1.60 (2H, quintet, J=7.4 Hz, H_2-3′), 2.02 (2H, m, H_2-15′), 2.11 (2×2H, m, H_2-11′, H_2-14′), 2.25 (2H, t, J=7.4 Hz, H_2-2′), 4.59 (1H, m, H-3), 4.91 (1H, apparent dd, J=10.2, *ca.* 0.5 Hz, H-17′a), 4.97 (1H, apparent dd, J=17.1, 1.7 Hz, H-17′b), 4.99 (1H, dd, J=15.1, 8.5 Hz, H-23), 5.13 (1H, dd, J=15.1, 8.5 Hz, H-22), 5.35 (1H, br d, J=*ca.* 4.0 Hz, H-6), 5.78 (1H, ddt, J=17.1, 10.2, 6.7 Hz, H-16′).

Hydrolysis of Compound 2 Compound **2** (0.7 mg) was stirred with NaOMe in absolute MeOH at 0 °C to ambient temperature for 30 min. Cold water was added; the mixture was neutralized with 5% HCl and extracted with $CHCl_3$. The organic phase was washed with water and the solvent was evaporated. The residue was chromatographed, eluted with hexane–$CHCl_3$ (from 99 : 1 to 97 : 3) to yield **1** and **4**. TLC comparison of the less polar component with authentic **4** revealed that they were the same compounds. The identity of the more polar component and compound **1** was made by TLC comparison and confirmed by the EI mass spectral comparison.

Hydrolysis of Compound 3 Compound **3** (1 mg) was similarly subjected to alkaline hydrolysis to yield **1** and **5**. The identity of these two hydrolysis products was confirmed in the same fashion as for compounds **1** and **4** described above.

Antiplasmodial Assay Antiplasmodial activity was evaluated against the parasite *Plasmodium falciparum* (K1, multidrug-resistant strain) that was cultured continuously according to the method of Trager and Jensen.[3] Quantitative assessment of antiplasmodial activity *in vitro* was determined by means of the microculture radioisotope technique based upon the method described by Desjardins *et al.*[4] The inhibitory concentration that caused a 50% reduction in parasite growth as indicated by *in vitro* uptake of [^{3}H]-hypoxanthine by *P. falciparum* was determined. An IC_{50} value of 1 ng/ml was observed for the standard drug, artemisinin, in the same test system.

Antimycobacterial Assay Antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H_{37}Ra using the Microplate Alamar Blue Assay.[5] In our system, the standard drugs, rifampicin, isoniazid, and kanamycin sulfate showed MIC values of 0.004, 0.06, and 2.5 μg/ml, respec-

tively.

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