Predomination of Dimers over Naturally Occurring Anthraquinones in Soil

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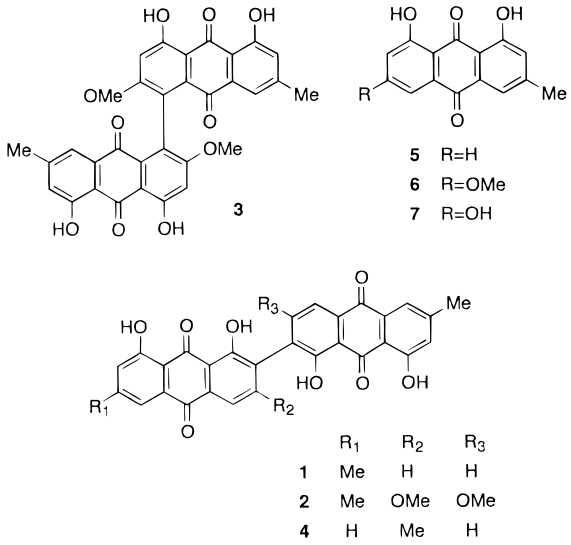
Four bianthraquinones and two monoanthraquinones were isolated as the major soil anthraquinones from a volcanic ash soil in Japan. They were identified as a new natural product 5,5′-biphyscion (named hinakurin) (**3**) and five known compounds, chrysotalunin (**1**), (−)-7,7′-biphyscion (**2**), microcarpin (**4**), chrysophanol (**5**), and physcion (**6**) using MS, 1D NMR, and 2D NMR techniques. Although the dimers (**1**−**4**) are rarely found as natural products, they, along with **5** and **6**, were ubiquitous and predominant over other anthraquinones in various soils from Japan and Nepal.

Nearly 400 anthraquinones have been isolated from natural biosources.[1] A kind of graveyard and/or a life stage of the whole terrestrial biosis, soil may be expected to contain almost any natural anthraquinone. The best-known soil anthraquinone, however, is a dimeric compound, chrysotalunin (7,7′-bichrysophanol, **1**), detected in soils throughout the world, but only from a soil source.[2−8] Recently, we found (−)-7,7′-biphyscion (**2**), a dimer anthraquinone that is structurally similar to **1**, which also occurs in soil samples from Japan.[9] Although **2** has been isolated from two toadstools in Europe,[10,11] this compound has not been found previously in Japan. Both dimers **1** and **2** are rarely found as natural products from biosources. Because **1** is frequently accompanied by other unidentified anthraquinones in soils,[4,5,12] we expected that soil samples would contain other rare anthraquinones. We now report the characterization of a new natural product (**3**) as well as the unusual chemical constitution and distribution of other anthraquinones found in various soils.



	R_1	R_2	R_3
1	Me	H	H
2	Me	OMe	OMe
4	H	Me	H

Results and Discussion

Six major soil anthraquinones were obtained from 15 kg of a volcanic-ash soil (Mt. Hinakura, Hyogo, Japan, Umbric Andosol) that was under vegetation, primarily *Sasa palmata* and *Pinus densiflora*. These anthraquinones were isolated by Soxhlet extraction with $CHCl_3$ and separated by repetitive chromatography and fractional crystallization. Two of the soil anthraquinones, compounds **3** and **4**, were obtained along with four known soil anthraquinones, chrysotalunin (**1**), (−)-7,7′-biphyscion (**2**), chrysophanol (**5**), and physcion (**6**). The structures of these compounds were elucidated using various spectroscopic methods.

Compound **3** was obtained as an orange-red microcrystal and exhibited λ_{max} 259, 300, and 455 nm consistent with a 1,8-dihydroxy-anthraquinone chromophore. The IR spectrum of **3** showed a weak shoulder at 1672 cm^{-1} and an intense band at 1626 cm^{-1} attributed to nonchelated and chelated carbonyl groups, respectively. Its molecular formula was determined to be $C_{32}H_{22}O_{10}$ by HREIMS (M^+ 566.1210). Reductive cleavage of **3** with alkaline sodium dithionite afforded only **6** (TLC and EIMS comparison), which indicates that compound **3** is a dehydrodimer of **6**. Moreover, the MS showed a molecular ion at *m*/*z* 566, which corresponds to a dehydrodimer of **6**; an ion at *m*/*z* 535, which may be formed by loss of methoxy from M^+; and an ion at *m*/*z* 283, which corresponds to **6**. The ^1H NMR of **3** showed two distinct peaks in the chelated hydroxyl region at δ 13.04 (2H, s) and 12.12 (2H, s); three aromatic proton signals at δ 7.33 (2H, br s, H-4, H-4′), 7.01 (2H, br s, H-2, H-2′), and 6.76 (2H, s, H-7, H-7′); one methoxy signal at δ 3.73 (6H, s, OCH_3-6, OCH_3-6′), and one methyl signal at δ 2.32 (6H, s, CH_3-3, CH_3-3′), indicating seven aromatic protons in the parent compound. The data from $^1H-^1H$ COSY experiments gave the four cross peaks (H-2, 2′ and H-4, 4′; H-2, 2′ and CH_3-3, 3′; H-4, 4′ and CH_3-3, 3′; and H-7, 7′ and OCH_3-6, 6′ proton). These data confirm compound **3** to be 1,1′,8,8′-tetrahydroxy-6,6′-dimethoxy-3,3′-dimethyl-5,5′-bianthraquinone, a new natural anthraquinone dimer named hinakurin (**3**). Hinakurin (**3**) was previously synthesized by methylation of skyrin, but the only analytical data reported was its elemental analysis and melting point.[13]

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Compound **4**, obtained as an orange microcrystal, exhibited UV, IR, and MS spectra identical with microcarpin.[14] The ^{1}H-NMR data of the acetylated **4** also corresponded to those of microcarpin tetraacetate. The ^{1}H-NMR spectra of **4** showed four distinct peaks in the chelated hydroxyl region at δ 12.44 (1H, s), 12.36 (1H, s), 12.00 (1H, s), and 11.92 (1H, s); eight aromatic proton signals at δ 7.97 (1H, d, J = 8.0 Hz, H-5′), 7.86 (1H, dd, J = 8.0, 1.1 Hz, H-5), 7.83 (1H, br s, H-4), 7.70 (1H, t, J = 8.0 Hz, H-6), 7.69 (1H, s, H-4′), 7.62 (1H, d, J = 8.0 Hz, H-6′), 7.31 (1H, dd, J = 8.0, 1.1 Hz, H-7), and 7.13 (1H, br s, H-2′); and two methyl signals at δ 2.49 and 2.30 (each 3H, s, CH_3-3, CH_3-3′). ^{1}H−^{1}H COSY experiments gave eight cross peaks (H-5′ and H-6′, H-5 and H-7, H-6 and H-7, H-5 and H-6, H-2′ and H-4′, H-2′ and CH_3-3′, H-4′ and CH_3-3′, and H-4 and CH_3-3 proton). Compound **4** was identified as 1,1′,8,8′-tetrahydroxy-3,3′-dimethyl-2,7′-bianthraquinone (microcarpin, 2,7′-bichrysophanol), first isolated from soil, from *Asphodelus microcarpus*[14] and the genus *Asphodeline*.[15−17]

The other known compounds, chrysotalunin (**1**) and (−)-7,7′-biphyscion (**2**), were identified by comparing their MS, HREIMS, and ^{1}H-NMR spectra and/or the spectra of their acetates with those reported in the literature.[3,11] Chrysophanol (**5**) and physcion (**6**) were identified by comparison of their TLC, IR, UV, EIMS, and ^{1}H-NMR data with authentic samples. These compounds (**1**, **2**, **5**, and **6**) were previously isolated from soil samples.

The distribution of these anthraquinones in various soils was examined by 2D TLC techniques.[5] Chloroform extracts of 25 surface soil samples (17 Umbric Andosols, 7 Distric Cambisols and a Haplic Acrisol) collected from various sites in Japan and Nepal, which ranged from subtropical to subarctic regions, were examined. The positive spots for hydroxyanthraquinones were confirmed by *in situ* recording of their UV spectra by densitometry and by spraying with KOH−MeOH and with AcOMg−MeOH. This procedure detects well-known anthraquinones such as the natural products **5** and **6**, and the four anthraquinone dimers (**1**−**4**). The other anthraquinones were also detected as positive spots by the spraying with 5% KOH−MeOH solution. The positive collection sites for the four dimers are illustrated in Figure 1.

Chrysophanol (**5**), physcion (**6**), and emodin (**7**) are widely distributed in many higher plants, soil fungi, and lichens, and such well-known anthraquinones would be expected to predominate in soil. Indeed, **5** and **6** were detected in most soil samples; however, emodin (**7**, an often predominant anthraquinone natural product[1]) was only detected in five soil samples; the other anthraquinones did not appear as positive spots. The EtOH extracts from several soil samples and their hydrolysates also gave similar results. Alternatively, four dimers (**1**−**4**) were detected ubiquitously in soils of Japan and Nepal (Figure 1). The predominant soil anthraquinones recognized thus far are dimers. Chrysotalunin (**1**) has been detected in a number of soils in Ireland,[2−4] Canada,[4] New Zealand,[6] and Japan,[5,7−9] and the other dimers (**2**−**4**) are also expected to be distributed throughout the world. Compound **1**, however, has thus far only been isolated from soil sources, and hinakurin (**3**) is a newly found natural compound. Although (−)-7,7′-biphyscion (**2**) and microcarpin (**4**) have been isolated from fungal sources (toadstools)[10,11] and from higher plants (Liliaceae),[14−17] respectively, these specific plants and fungi are not found in our sampling sites (or in Japan and Nepal). According to Steglich and co-workers, **2**, isolated from the toadstools *Dermocybe cinnamomeolutea* and *Tricholoma equestre*,[10,11] was probably an artifact derived from a flavomannin-type precursor, and there are many reports of flavomannin- or atrovirin-type compounds as coupled pre-anthraquinones.[18]

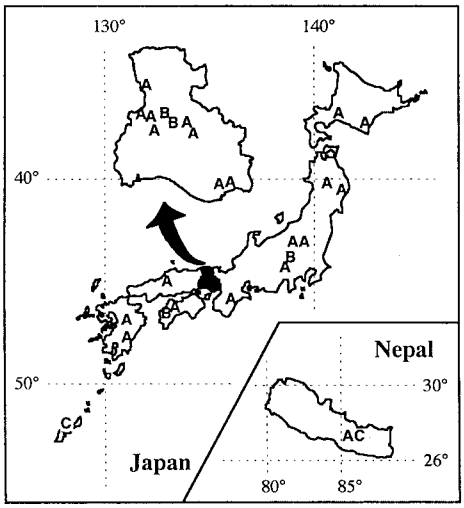

Figure 1. Collection sites for anthraquinone-dimer positive soil samples from Japan and Nepal. The lettering on the maps indicate sites positive for **A** (**1**−**4**), **B** (**2**−**4**), and **C** (**3** and **4**).

The origin of the four dimer anthraquinones (**1**−**4**) in soil is unknown. It is possible, however, that these dimeric anthraquinones or their precursors are common compounds produced directly by a biosource (possibly microorganisms) under natural conditions, because such anthraquinones are ubiquitously found in soils under quite different environments (e.g., vegetation, soil type, and climate). Otherwise, the four predominant dimers may be formed in soil via some unidentified dimerization reaction, as they consist only of monomers **5** and **6**. In any case, our results indicate that further studies are needed for more complete understanding of the origin of these anthraquinone compounds.

Experimental Section

General Experimental Procedures. The ^{1}H- and COSY NMR spectra were recorded on a Bruker DPX 250 spectrometer in $CDCl_3$. COSY-45 was obtained using standard Bruker software. Chemical shifts were referenced relative to $CHCl_3$ (7.24 ppm) or to an external TMS standard. UV spectra were recorded with a JASCO V-530 spectrophotometer, and IR spectra were recorded using a Shimadzu FTIR-4000 spectrophotometer. EIMS and HREIMS were obtained using a Hitachi 4100 instrument (70 eV). Optical rotations were measured with a JASCO ORD/UV-5 polarimeter. Column chromatography was performed on Wakogel C-200 (Wako) and Cosmosil 75 C_{18}-open (Nakarai Tesque). TLC analysis was performed on Kieselgel 60G F_{254} (Merck).

Soil Materials. For the isolation of anthraquinones, a soil sample belonging to Umbric Andosol (volcanic-ash soil) was taken from the top 20-cm horizon at Mt. Hinakura in Hyogo prefecture, Japan, an area that was

under vegetation (primarily *Sasa palmata* and *Pinus densiflora*.). For the 2D TLC test for anthraquinones, 25 of the surface soil samples (top 10−20 cm horizon) belonging to Umbric Andosols (17 soils), Distric Cambisols (7 soils), and Haplic Acrisol (1 soil) were collected from locations ranging from the subtropical to the subarctic region in Japan, and two of the surface soil samples (top 10-cm horizon) belonging to Distric Cambisols were collected in Nepal. These sampling sites are indicated in Figure 1. The sampling sites in Japan were areas under various vegetation (e.g., deciduous broad-leaf forests, evergreen broad-leaf forests, needle-leaf forests, Sasa glasslands, or Miscanthus glassland).[8] One of the sampling sites in Nepal was an area under pasture of grazing grass and another was pine forests (*Pinus wallichiana*). Each soil was air-dried and passed through a 2-mm screen.

Extraction and Isolation. Air-dried soil samples (15 kg) were extracted with hot $CHCl_3$ for 1 day. The $CHCl_3$ extract (45 L), including precipitate, was filtered through a membrane filter (1.0 μm). The precipitate (27 g) on the filter was successively washed with MeOH, H_2O, Me_2CO, EtOAc, and *n*-hexane and repeatedly recrystallized from $CHCl_3$, and 70 mg of **1** was obtained. The concentrated syrupy residue (84 g), obtained by removal of the solvent from the filtrates and washings *in vacuo*, was partitioned between $CHCl_3$ and H_2O. The former layer was concentrated under reduced pressure, and the resulting syrupy residue was chromatographed on Si gel (600 g) using $CHCl_3$ as eluent. The eluate (23 g) was concentrated and rechromatographed on Si gel (200 g) by eluting with a stepwise gradient of hexane−C_6H_6 (1:1) to C_6H_6. Individual fractions were combined (TLC monitoring) to provide three positive hydroxyanthraquinone fractions. The first fraction (1.4 g) was subjected to preparative TLC (Si gel, 2 mm, petroleum benzine−EtOAc−formic acid−H_2O 89:10:1:saturated) and yielded an upper yellow band (**5**, $R_f = 0.55$, 15 mg) and a lower yellow band (**6**, $R_f = 0.33$, 18 mg). The second fraction (2.6 g) was dissolved in CCl_4, and the CCl_4 solution was filtered through a membrane filter (0.2 μm). The concentrated filtrate was rechromatographed on preparative TLC (Si gel, 2 mm, hexane−Me_2CO−H_2O 10:10:7), and the eluate from the major yellow band was successively recrystallized from EtOH, hexane, and MeCN to produce **4** (11 mg). The third fraction (2.7 g) was subjected to column chromatography (Cosmosil 75 C_{18}-open) with 75% EtOH. The first yellow band on a C_{18} column was then collected, concentrated, and rechromatographed on preparative TLC (Si gel, 2 mm, hexane−EtOAc−H_2O 9:1:saturated). The major yellow band ($R_f = 0.45$) on preparative TLC was eluted with EtOH, and the eluate was recrystallized from EtOH (**3**, 8 mg). The second yellow band on the C_{18} column was collected, and the eluate was recrystallized from EtOAc (**2**, 15 mg).

Chrysotalunin (1): orange amorphous solid; HREIMS 506.1011 (calcd for $C_{30}H_{18}O_8$, 506.1000); $[\alpha]_D$ 0° ($CHCl_3$); other spectroscopic data (EIMS, UV, IR) were in agreement with literature data.[3]

Acetylation of 1. A mixture of compound **1** (15 mg), Ac_2O (1 mL), and pyridine (2 mL) was heated in a boiling H_2O bath for 30 min. After evaporation *in vacuo*, the reaction residue was subjected to preparative TLC on Si gel ($CHCl_3$−EtOAc (9:1), $R_f = 0.43$) to obtain chrysotalunin tetraacetate (light yellow powder, 9 mg); UV, ν_{max} (IR), EIMS, and ^{1}H-NMR (250 MHz, $CDCl_3$) data agreed with the literature.[3]

(−)-7,7′-Biphyscion (2): orange microcrystals; HREIMS 566.1142 (calcd for $C_{32}H_{22}O_{10}$, 566.1211); UV, ORD, ν_{max} (IR), EIMS, and ^{1}H-NMR (250 MHz, CCl_4) data in agreement with literature data.[10]

Acetylation of 2. A mixture of compound **2** (5 mg), Ac_2O (0.4 mL) and pyridine (0.8 mL) was heated in a boiling H_2O bath for 30 min. After evaporation *in vacuo*, the reaction residue was chromatographed by preparative TLC on Si gel ($CHCl_3$−EtOAc 9:1, $R_f = 0.43$) to obtain chrysotalunin tetraacetate (light yellow powder, 3 mg): UV, ν_{max} (IR), EIMS, and ^{1}H-NMR (250 MHz, $CDCl_3$) data agreed with the literature.[11]

Hinakurin (3): orange-red microcrystals; UV ($CHCl_3$) λ (log ϵ) 259 (4.59), 300 (4.40), 455 (4.28) nm; IR (KBr) ν_{max} 1672 (nonchelated C═O), 1626 cm^{-1} (chelated C═O); $[\alpha]_D$ 0° ($CHCl_3$); HREIMS 566.1211 (calcd for $C_{32}H_{22}O_{10}$, 566.1211); EIMS *m/z* (rel int): 566 [M^+] (100), 535 (28), 504 (9), 297 (17), 283 (11); ^{1}H NMR (250 MHz, $CDCl_3$) δ 13.04 (2H, s), 12.12 (2H, s), 7.33 (2H, br s, H-4, H-4′), 7.01 (2H, br s, H-2, H-2′), 6.76 (2H, s, H-7, H-7′), 3.73 (6H, s, OCH_3-6, OCH_3-6′), 2.32 (6H, s, CH_3-3, CH_3-3′).

Cleavage of 3. A mixture of compound **3** (2 mg), 1N NaOH (2 mL), and $Na_2S_2O_4$ (0.1 g) was heated at 70 °C for 20 min. After extraction with $CHCl_3$ followed by acidification with HCl, the $CHCl_3$ layer was evaporated *in vacuo*. The residue was chromatographed on a TLC plate by developing with petroleum ether−EtOAc−H_2O (75:25:3.5). The major compound was identified as **5** by co-TLC and EIMS data by comparison with an authentic sample.

Microcarpin (4): orange microcrystals; HREIMS 506.0996 (calcd for $C_{30}H_{18}O_8$, 506.1000); ^{1}H NMR (250 MHz, $CDCl_3$) δ12.44 (1H, s), 12.36 (1H, s), 12.00 (1H, s), 11.92 (1H, s), 7.97 (1H, d, J = 8.0 Hz, H-5′), 7.86 (1H, dd, J = 8.0, 1.1 Hz, H-5), 7.83 (1H, br s, H-4), 7.70 (1H, t, J = 8.0 Hz, H-6), 7.69 (1H, s, H-4′), 7.62 (1H, d, J = 8.0 Hz, H-6′), 7.31 (1H, dd, J = 8.0, 1.1 Hz, H-7), 7.13 (1H, br s, H-2′), 2.49 (3H, s, CH_3-3′), 2.30 (3H, s, CH_3-3); assignments are based on interpretation of COSY NMR data, and UV, ORD, ν_{max} (IR), and EIMS data in close agreement with literature data.[14] ^{1}H-NMR data are reported for the first time.

Acetylation of 4. A mixture of compound **4** (1.5 mg), Ac_2O (0.05 mL), and pyridine (0.1 mL) was heated at 60 °C for 30 min. After evaporation *in vacuo*, the reaction residue was subjected to preparative TLC on Si gel ($CHCl_3$, $R_f = 0.55$) to obtain microcarpin tetraacetate (orange yellow powder, < 1 mg): UV, ν_{max} (IR), EIMS, and ^{1}H-NMR (250 MHz, $CDCl_3$) data in close agreement with the literature.[14]

Chrysophanol (5): obtained as an orange-yellow powder and identified by comparison of its TLC, IR, UV, EIMS, and ^{1}H-NMR data with an authentic sample.

Physcion (6): obtained as orange needles and identified by comparison of its TLC, IR, UV, EIMS, and ^{1}H-NMR data with an authentic sample.

Detection of Soil Hydroxyanthraquinones. Soil samples (20 g) were extracted with $CHCl_3$ using Soxhlet.

The $CHCl_3$-extract solution was washed with distilled H_2O and evaporated to a small volume. The concentrated solution was applied to a mini Si gel column, and impurities were removed by elution with hexane. A portion of the fraction that eluted with hexane−EtOAc (3:1) was applied to the 2D TLC of Si gel plate.[5] The positive spots for hydroxyanthraquinones were confirmed by *in situ* recording of UV spectra by densitometry and by spraying with KOH−MeOH and with AcOMg−MeOH.

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