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Subulatin, an Antioxidic Caffeic Acid Derivative Isolated from the *In Vitro* Cultured Liverworts, *Jungermannia subulata*, *Lophocolea heterophylla*, and *Scapania parvitexta*

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The new caffeic acid derivative, subulatin (1), was isolated from *in vitro* cultured liverworts, *Jungermannia subulata*, *Lophocolea heterophylla*, and *Scapania parvitexta*. The structure of 1 involved two caffeic acids, D-glucose, and 2-carboxy-6-(1,2-dihydroxy-ethyl)-4,5-dihydroxy-5,6-dihydro-4*H*-pyran. The connectivity of those and the absolute stereochemistry of 1 were elucidated on the basis of spectroscopic evidence. The antioxidative activity of 1 was comparable to that of α-tocopherol. (2′*R*)-Phaselic acid (2a) and (−)-9,2″-epiphylloyl-L-malic acid (4) were also isolated from *J. subulata* and *L. heterophylla*, respectively. A chiral HPLC analysis of the *p*-bromobenzoyl-malic acids derived from 2a showed that 2a from *J. subulata* was unusual (+)-*trans*-caffeoyl-D-malic acid.

Key words: Hepaticae; antioxidant; lignan; caffeic acid; phaselic acid

Bryophytes are believed to be among the oldest of land plants. An investigation of the chemical constituents of Bryophytes has shown the presence of unique metabolites.[1)] For example, a naphthalene derivative (epiphyllic acid, **3**) has been isolated from gametophytes of *Pellia epiphylla*.[2,3)] Further oxygenated lignan derivatives, scapaniapyrone,[4)] jamesopyrone[5)] and erimopyrone,[6)] have been isolated from *Scapania undulata*, *Jamesoniella autumnalis*, and *Moerckia erimona*, respectively. These are biosynthesized from two caffeic molecules, a common phenolic unit in liverworts.[7)] Unusual polyoxyganated hydrocarbons which conjugated with caffeic acids or lignans have recently been isolated from some liverworts.[8,9)] Interest in the biosynthesis of these compounds and continuation of our study on the presence and structural variation of unique phenylpropanoids in liverworts prompted us to examine the three liverworts, *Jungermannia subulata*, *Lophocolea heterophylla* and *Scapania parvitexta*, all belonging to the order Jungermanniales. An earlier work on *J. subulata* has demonstrated the growth behavior of cells[11,12)] and the production of *ent*-kaurene by a cell suspension culture.[13)] We have recently isolated *ent*-kaurane-3,15-dione together with three known *ent*-kaurane-type diterpenes.[10)] Gametophytes of *L. heterophylla* have a typical mossy aroma,[14)] and homoborneol and oxygenated sesquiterpenes have recently been isolated from *L. heterophylla*.[15)] *S. parvitexta* has been analyzed for its lipophilic constituents by a GC-MS analysis.[16)]

We present in this paper the isolation and structural elucidation of a new antioxidative compound named subulatin (**1**) from the *in vitro* cultured liverworts, *J. subulata*, *L. heterophylla*, and *S. parvitexta*. We also discuss the enantiomeric composition of malic acid esterified with epiphyllicacid (**3**) or caffeic acid in liverworts from a chiral HPLC analysis.

Experimental

General. NMR spectra were recorded in CD_3OD, CD_3CN, $(CD_3)_2SO$ and D_2O solns, using Jeol EX-270 (H, 270 MHz; C, 68.5 MHz) and Bruker AMX500 (H, 500 MHz; C, 125 MHz) instruments relative to CH_3OH at δ_H 3.35, CH_3CN at δ_H 1.90, CD_3OD at δ_C 49.8, and CD_3CN at δ_C 108.0. ^{13}C multiplicity was determined by using the DEPT pulse sequence. EI-MS data were measured by a Jeol JMS-AX500 instrument, and FAB-MS data were meas-

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ured by a Jeol JMS-SX102A instrument. IR spectra were taken with a Jasco FT/IR-420 instrument by the KBr disk method. Optical rotation values were recorded with a Jasco DIP-370 polarimeter in MeOH. UV spectra were measured with a Shimadzu UV-1600 instrument in MeOH, and CD spectra with a Jasco J-725 instrument in MeOH. Antioxidative activity was measured by the erythrocyte membrane ghost system.[17]

Collection and in vitro culture of the liverworts. The collection and culture conditions for *J. subulata* and *L. heterophylla* have been previously reported.[7,10] *S. parvitexta* was collected from the bank of the Satsunai river (Hokkaido, Japan) in 1997. *S. parvitexta* was identified by T. F., and a voucher specimen has been deposited at the Department of Bioresource Science in Obihiro University of Agriculture and Veterinary Medicine. *In vitro* culture of the liverwort, *S. parvitexta* was initiated from spores, and callus induction was achieved on an MSK-4 medium with 4% glucose.

Extraction and isolation. Dried plant material of *J. subulata* (51.0 g) was milled and extracted with MeOH (500 ml × 3). The combined MeOH extract was evaporated under reduced pressure and suspended in H_2O (200 ml). The solution was washed with Et_2O (200 ml × 2) and extracted with *n*-BuOH (200 ml × 3). The combined *n*-BuOH extract was evaporated to dryness and passed through a Bond Elut C18 cartridge with MeCN–H_2O (1:1) as the eluent. The MeCN–H_2O (1:1) eluate was separated by HPLC (Daiso SP-120-5-ODS-BP, 20 mm i.d. × 250 mm, 0.1% HCOOH-20% MeCN/H_2O) to afford **1** (50.4 mg) and **2a** (97.6 mg).

Dried plant material of *L. heterophylla* (67.0 g) was milled and extracted with MeOH (500 ml × 3). The combined MeOH extract was evaporated to dryness (25.1 g) under reduced pressure and suspended in H_2O (700 ml). The solution was washed with Et2O (700 ml × 3) and extracted with *n*-BuOH (700 ml × 3). The combined *n*-BuOH extract was evaporated to dryness (2.0 g) and passed through Bond Elut C18 cartridge with MeCN–H_2O (1:1) as the eluent. The MeCN–H_2O (1:1) eluate was separated by HPLC (Daiso SP-120-5-ODS-BP, 20 mm i.d. × 250 mm, 0.1% HCOOH-20% MeCN/H_2O) to afford **1** (12.0 mg). Further purification by HPLC (Daiso SP-120-5-ODS-BP, 20 mm i.d. × 250 mm, 0.1% HCOOH-10% MeCN/H_2O) gave **3** (70.6 mg) and **4** (94.3 mg).

Dried plant material of *S. parvitexta* (34.6 g) was milled and extracted with MeOH (300 ml × 3). The combined MeOH extract was evaporated to dryness (5.8 g) under reduced pressure and suspended in H_2O (200 ml). The solution was washed with Et2O (200 ml × 3) and extracted with *n*-BuOH (300 ml × 3). The combined *n*-BuOH extract was evaporated to dryness (1.8 g) and passed through a Bond Elut C18 cartridge with MeCN–H_2O (1:1) as the eluent. The MeCN–H_2O (1:1) eluate was separated by HPLC (Daiso SP-120-5-ODS-BP, 20 mm i.d. × 250 mm, 0.1% HCOOH-20% MeCN/H_2O) to afford **1** (0.5 mg).

Subulatin (1). Pale yellow amorphous powder, $[\alpha]_D$ −16.8°(MeOH, *c* 0.19). FABMS *m/z*: 746 $[M+K]^+$, 729 $[M+Na]^+$, 707 $[M+H]^+$. UV λ_{max} (MeOH) nm (log ε): 209 (4.39), 213 (4.43), 218 (4.43), 237 (4.31), 294 (4.38), 325 (4.38). IR ν^{KBr} cm^{-1}: 3388, 1708, 1597, 1279. CD: $\Delta\varepsilon$ 213 +16.1, $\Delta\varepsilon$ 250 −9.7, $\Delta\varepsilon$ 266 −1.6, $\Delta\varepsilon$ 287 −10.7, $\Delta\varepsilon$ 339 +20.4 (MeOH; *c* 1.13 × 10^{-5}). ^{1}H-NMR and ^{13}C-NMR: see Tables 1 and 2, respectively.

(2′R)-Phaselic acid (2a). Pale yellow amorphous powder, $[\alpha]_D$ −37.7°(H_2O, *c* 2.18). HR-FABMS $[M-H]^-$ *m/z*: calcd. for $C_{13}H_{11}O_8$, 295.04570; found, 295.04539. UV λ_{max} (MeOH) nm (log ε): 220(4.09), 245 (4.30), 330 (4.38). IR ν^{KBr} cm^{-1}: 3365, 2933, 1720, 1630, 1605, 850. ^{1}H-NMR δ_H (CD_3OD): 3.07 (2H, *m*, H-3′), 5.48 (1H, *t*, *J*=5.5 Hz, H-2′), 6.19 (1H, *d*, *J*=16.0 Hz, H-8), 6.81 (1H, *d*, *J*=8.3 Hz, H-5), 6.90 (1H, *d*, *J*=8.3 Hz, H-6), 7.00 (1H, *s*, H-2), 7.46 (1H, *d*, *J*=16.0 Hz, H-7). ^{13}C-NMR δ_C (CD_3OD): 37.8 (C-3′), 70.7 (C-2′), 114.9 (C-8), 115.9 (C-2), 117.3 (C-5), 124.0 (C-6), 128.4 (C-1), 147.5 (C-4), 148.7 (C-7), 150.5 (C-3), 168.9 (C-1′, C-4′), 173.9 (C-9).

(−)-9,2″-Epiphylloyl-L-malic acid (4). Colorless syrup, $[\alpha]_D$ −61.0° (MeOH, *c* 1.42). FABMS *m/z*: 746 $[M-H]^-$. UV λ_{max} (MeOH) nm (log ε): 205 (4.14), 222 (3.95), 253 (3.90), 310 (3.54), 337 (3.57). IR ν^{KBr} cm^{-1}: 3363, 2936, 1715, 1645, 1190, 669. ^{1}H-NMR δ_H (CD_3OD): 2.90 (2H, *d*, *J*=6.3 Hz, H-3″), 3.86 (1H, *d*, *J*=2.3 Hz, H-2), 4.41 (1H, *d*, *J*=2.3 Hz, H-1), 5.43 (1H, *t*, *J*=6.3 Hz, H-2″), 6.40 (1H, *d*, *J*=8.3 Hz, H-6′), 6.42 (1H, *s*, H-2′), 6.55 (1H, *s*, H-8), 6.62 (1H, *d*, *J*=8.3 Hz, H-5′), 6.85 (1H, *s*, H-5), 7.65 (1H, *s*, H-4). ^{13}C-NMR δ_C (CD_3OD): 37.1 (C-3″), 46.9 (C-1), 47.8 (C-2), 70.2 (C-2″), 115.7 (C-2′), 116.2 (C-5′), 117.1 (C-5), 117.2 (C-8), 119.8 (C-6′), 122.2 (C-3), 124.8 (C-1′), 131.5 (C-4a), 136.3 (C-8a), 140.4 (C-4), 144.8 (C-4′), 145.5 (C-7), 145.9 (C-3′), 149.1 (C-6), 167.6 (C-9), 173.0 (C-4″), 173.8 (C-1″), 176.2 (C-10).

Enzymatic hydrolysis of 1. 5 mg of compound **1** was suspended in a 0.1 M sodium acetate buffer (pH 5.9, 5 ml). The reaction was initiated by adding 1 mg of β-glucosidase (Toyobo, 11.6 U/mg). After incubating for 30 min at 30°C, the solution was subjected to a TLC analysis with authentic D-glucose (R_f 0.42, SiO_2, EtOAc-HCOOH-AcOH–H_2O

(100:11:11:27) as the solvent system).

Isolation of (2′S)-phaselic acid (2b) from red clover (Trifolium pratense). Dried plant material of *T. pratense* (100 g) was milled and extracted with MeOH (600 ml × 2). The combined MeOH extract was concentrated *in vacuo* and dissolved in H_2O (400 ml). The H_2O solution was washed with $(C_2H_5)_2O$ (400 ml × 3) and then extracted with EtOAc (400 ml × 3). The combined EtOAc extract was concentrated *in vacuo* and separated by HPLC (Daiso SP-120-5-ODS-BP, 20 mm i.d. × 250 mm, 0.1% HCOOH-20% MeCN/H_2O) to afford **2b** (5.3 mg) as pale yellow amorphous powder, $[\alpha]_D$ +39.3°(H_2O, *c* 0.53).

Preparation of p-bromobenzoyl-L-malic acid (5b) and p-bromobenzoyl-DL-malic acid (5c). A $(C_2H_5)_2O$ solution of CH_2N_2 was added a flask containing L-malic acid (100 mg), and the mixture stood for 10 hrs. The reaction mixture was evaporated *in vacuo* to afford a colorless oil (86.3 mg) and then dissolved in $(C_2H_5)_2O$ (15 ml) together with DCC (309.7 mg), *p*-bromobenzoic acid (375.7 mg) and DMAP (193.1 mg). After 1 hr, the solution was subjected into SiO_2 column chromatography (Wako gel C-200, 10 g; *n*-hexane:EtOAc = 9:1) to afford **5b** (91.6 mg, 12.1%) as white powder. HR-EI-MS $[M]^+$ *m/z*: calcd. for $C_{13}H_{13}O_6Br$, 343.9896; found, 343.9919. EI-MS *m/z* (rel. int.): 346 (10), 344 (11), 287 (5), 285 (5), 185 (97), 183 (100), 113 (8), 104 (6). UV λ_{max} (MeOH) nm (log ε): 207 (3.87), 245 (4.03). IR ν^{KBr} cm^{-1}: 2954, 1724, 1589, 753. ^{1}H-NMR δ_H ($CDCl_3$): 3.01 (2H, *d*, *J* = 6.8 Hz, H-3′), 3.70 (3H, *s*, COO*Me*), 3.76 (3H, *s*, COO*Me*), 5.60 (1H, *t*, *J* = 6.3 Hz, H-2′), 7.56 (2H, *d*, *J* = 7.9 Hz, H-2, H-6), 7.88 (2H, *d*, *J* = 7.9 Hz). *p*-Bromobenzoyl-DL-malic acid (**5c**) was prepared in a similar manner.

Preparation of p-bromobenzoyl-malic acids (5 s) from phaselic acids (2 s) and (−)-9,2″-epiphylloyl-L-malic acid (4). (2′*R*)-Phaselic acid (**2a**, 11.3 mg isolated from *J. subulata*) was dissolved in a 2*N*–KOH solution and stirred for 4 hrs under N_2 gas. The reaction mixture was acidified to pH 3.5 with 2*N*–HCl and then extracted with EtOAc. The EtOAc extract was concentrated *in vacuo*, a $(C_2H_5)_2O$ solution of diazomethane was added, and the mixture stood for 10 hrs. The reaction mixture was evaporated *in vacuo* and then dissolved in $(C_2H_5)_2O$ (1.6 ml) together with DCC (30.6 mg), *p*-bromobenzoic acid (37.3 mg) and DMAP (19.3 mg). After 1 hr, the solution was subjected SiO_2 column chromatography (Wako gel C-200, 500 mg; *n*-hexane:EtOAc = 9:1) to afford **5a** (2.6 mg). **5b** from **2b** isolated from *T. pratense* and from **4** isolated from *L. heterophylla* were prepared as the same manner to afford 1.0 mg and 1.1 mg, respectively.

Chiral HPLC analysis of 5 s. The enantiomeric compositions of **5** s were determined by HPLC, using a chiral column (Waters Opti-Pak XC, 300 × 3.9 mm) and UV detector at 254 nm. The mobile phase was *n*-hexane-EtOH (4:1) at a flow of 1 ml min^{-1} to separate **5b** (L form, R_t 9.79 min) and **5a** (D form, R_t 7.17 min). The average ratio of the D-isomer to the L-isomer was estimated on the basis of their relative peak areas in consideration of the relative peak area of a 1:1 mixture of the D- and L-isomers.

Results and Discussion

The *n*-BuOH-soluble portion of the MeOH extract of the *in vitro* cultured liverwort, *J. subulata*, was separated by HPLC (ODS) to afford compounds **1** (50.4 mg) and *2a* (97.6 mg). The *n*-BuOH-soluble portion of the MeOH extract of the *in vitro*-cultured liverwort, *L. heterophylla*, was separated by HPLC (ODS) to afford compounds **1** (12.0 mg), **3** (70.6 mg) and **4** (94.3 mg). Compound **1** (0.5 mg) was also isolated from the *in vitro*-cultured liverwort, *S. parvitexta*.

Compound **1**, a colorless syrup, gave the $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ion peaks at *m/z* 707, 729 and 745 in the FAB^+ mass spectrum corresponding to a molecular weight of 706. The ^{13}C-NMR spectrum (in CD_3OD) containing the signals of two methylenes, twenty methines and six quaternary carbons indicated the presence of two carboxyl groups (δ_C 168.6 and 167.5) and seventeen aromatic carbons (δ_C 149.8 − 107.5). The ^{13}C-NMR spectrum of compound **1** in CD_3CN-D_2O (3:1) showed an additional olefinic quaternary carbon at δ_C 146.0. The ^{1}H-NMR spectrum (in CD_3OD) displayed the signals for 23 protons which contained two pairs of doublets (δ_H 7.55, 7.49, 6.37 and 6.24), six protons for two sets of 1,3,4-trisubstituted benzenes, a broad singlet containing three protons at δ_H 5.88 and ten protons overlapping at δ_H 4.25 − 3.42. DQF ^{1}H–^{1}H COSY and HSQC experiments indicated that a remaining signal overlapped with an HDO signal (δ_H 4.75) which was correlated with the methine carbon at δ_C 103.4. The extensive DQF ^{1}H–^{1}H COSY, TOCSY, HSQC and HMBC experiments in both CD_3OD and CD_3CN-D_2O (3:1) revealed that compound **1** consisted of four partial structures. Two of those were identified as caffeic acid. The third moiety was β-D-glucose which had been liberated from the hydrolysis of compound **1** with β-glucosidase. The remaining moiety consisted of a trisubstituted double bond (δ_C 146.0 C-2 and δ_C 105.4 C-3) and five oxygen-bearing aliphatic carbons, (δ_C 65.1 C-4, δ_C 61.2 C-5, δ_C 74.7 C-6, δ_C 67.8 C-7 and δ_C 61.4 C-8). These seven carbon signals and their correlated proton signals in the ^{1}H-NMR spectrum are very similar to those of the C8 moiety (2-carboxy-6-(1,2-dihydroxy-ethyl)-4,5-dihydroxy-5,6-dihydro-4*H*-pyran) in pelliatin, except

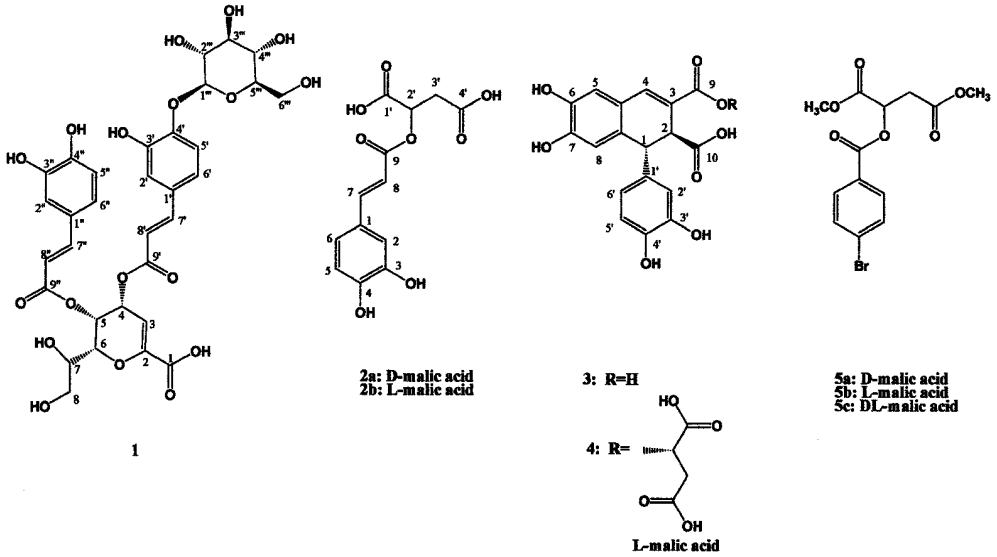


Fig. 1. Compounds Isolated from the Liverworts.

Table 1. ^{1}H-NMR Data for Compound **1**

H-no.	Chemical shift (ppm)		*J* in Hz	Chemical shift (ppm)		*J* in Hz
	CD_3OD			CD_3CN+D_2O (3:1)		
3	5.82	*s*		5.79	*m*	
4	5.88	*s*		5.79	*m*	
5	5.88	*s*		5.73	*m*	
6	4.25	*d*	*J*=8.9	4.19	*d*	*J*=7.9
7	3.78	*dd*	*J*=3.5, 8.9	3.71	*m*	
8	3.83	*brs*		3.73	*m*	
2′	7.05	*d*	*J*=2.0	7.02	*d*	*J*=2.0
5′	7.04	*d*	*J*=7.3	6.78	*d*	*J*=8.1
6′	6.84	*dd*	*J*=2.0, 7.3	6.93	*dd*	*J*=2.0, 8.1
7′	7.49	*d*	*J*=16.0	7.47	*d*	*J*=16.0
8′	6.24	*d*	*J*=16.0	6.32	*d*	*J*=16.0
2″	7.01	*d*	*J*=1.7	7.04	*d*	*J*=2.0
5″	6.77	*d*	*J*=8.4	6.92	*d*	*J*=8.4
6″	6.92	*dd*	*J*=1.7, 8.4	6.81	*dd*	*J*=2.0, 8.4
7″	7.55	*d*	*J*=15.9	7.42	*d*	*J*=16.0
8″	7.37	*d*	*J*=15.9	6.21	*d*	*J*=16.0
1‴	4.81	*m*		4.83	*M*	
2‴	3.50	*m*		3.46	*M*	
3‴	3.49	*m*		3.46	*m*	
4‴	3.43	*m*		3.37	*m*	
5‴	3.44	*m*		3.44	*ddd*	*J*=2.2, 5.0, 7.4
6‴	3.72	*m*		3.63	*dd*	*J*=2.2, 12.3
	3.88			3.74	*dd*	*J*=5.0, 12.3

Table 2. ^{13}C-NMR Data for Compound **1**.

C-no.	Chemical shift (ppm)	Chemical shift (ppm)
	CD_3OD	CD_3CN+D_2O (3:1)
1	*	*
2	*	146.0
3	107.5	105.4
4	67.4	65.1
5	63.5	61.2
6	77.2	74.7
7	70.2	67.8
8	64.0	61.4
1′	130.8	128.3
2′	115.6	113.7
3′	148.5	145.6
4′	149.0	146.3
5′	117.9	115.3
6′	122.8	121.2
7′	146.8	144.6
8′	116.6	114.6
9′	167.5	165.5
1″	127.7	125.5
2″	115.4	113.9
3″	146.8	144.0
4″	149.8	146.9
5″	116.6	114.8
6″	123.2	121.4
7″	148.1	145.8
8″	114.5	112.8
9″	168.6	166.4
1‴	103.4	100.3
2‴	74.8	72.0
3‴	77.5	74.6
4‴	71.3	68.5
5‴	78.3	75.4
6‴	62.4	59.8

* No signals were detected.

for the signal of a carboxylic carbon.[3] These observations together with the molecular weight prompted us to deduce the molecular formula of compound **1** as $C_{32}H_{34}O_{17}$, and two caffeic acids, β-D-glucose and the C8 moiety in the molecule were connected by three ester or ether linkages. The ROESY and HMBC data established the connectivity of this moiety in compound **1**. The overlapping signals due to H-4 and H-5 (δ_H 5.88 in CD_3OD) were resolved in CD_3CN/D_2O (3:1). The HMBC experiment on compound **1** in CD_3CN/D_2O (3:1) revealed CH long-range coupling between H-4 and C-9′, between H-5 and C-9″, and between H-1‴ and C-4′. ROE signals between H-5′

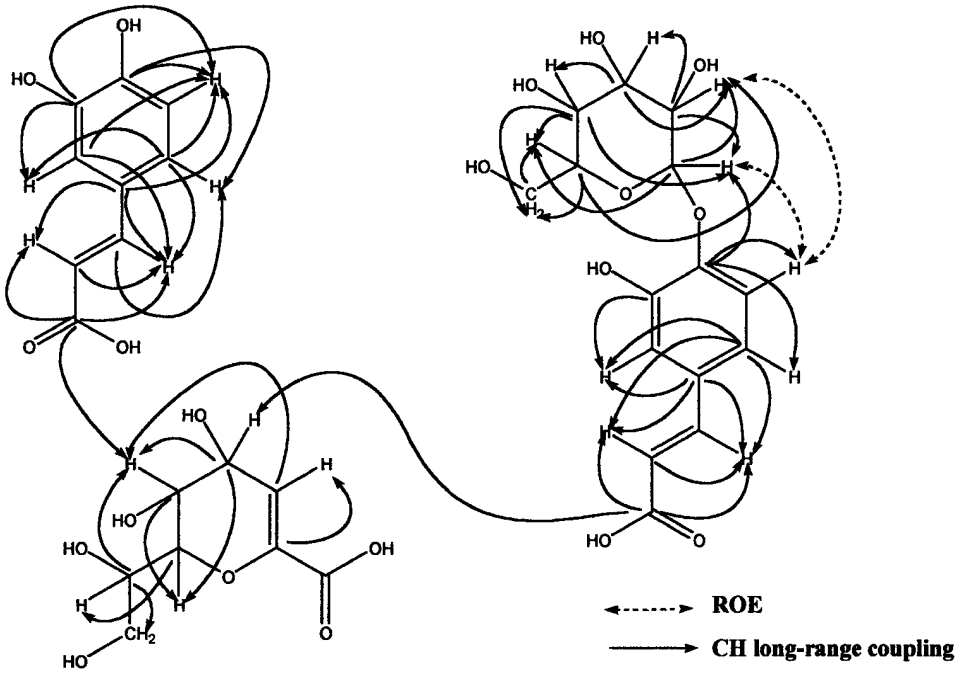


Fig. 2. Significant HMBC Correlations in Compound **1**.

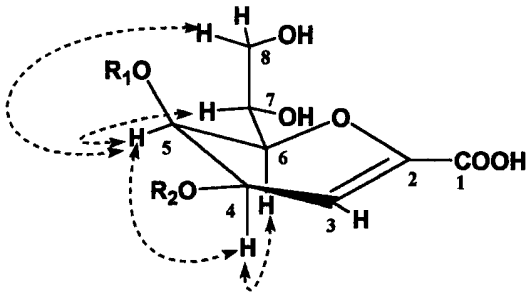
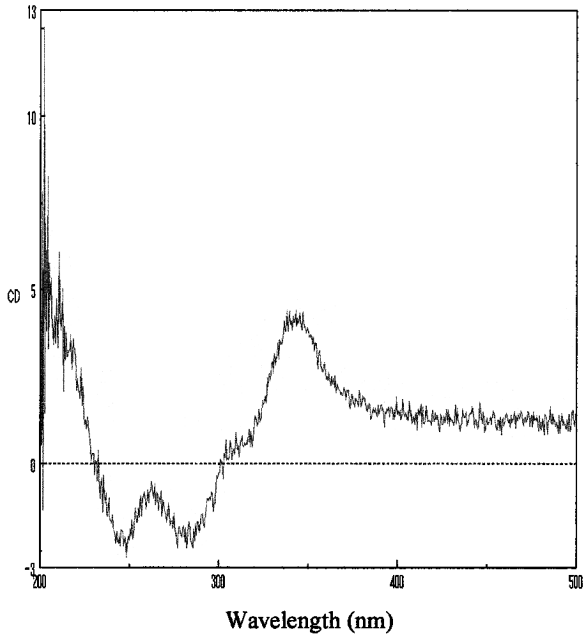


Fig. 3. Significant ROEs of the C8 Moiety in Compound **1**.

and H1‴ and between H-5′ and 2‴ were also observed. Thus, the connectivity of four partial structures was established as shown in Fig. 2. The ROESY experiment on **1** indicated that the relative stereochemistry of the C8 alcohol moiety in **1** was the same as that of pelliatin.[3] CD spectrum was measured to identify the absolute stereochemistry of **1**. Compound **1** showed a negative Cotton effect at 287 nm and a positive Cotton effect at 339 nm. The structure of **1** has 1,2-dicaffeoyl at C-4 and C-5 of the C8 alcohol moiety as shown in Fig. 4. The CD spectrum of **1** was thus expected to show a similar Cotton effect with those chromophores like 1,2-dibenzoates, suggesting that the configuration of **1** might be 4*R*,5*R*. Compound **1** isolated from the three liverworts, *J. subulata*, *L. heterophylla*, and *S. parvitexta*, gave the same results, and it was named subulatin. Similar C8 alcohols have recently been isolated from the liverwort, *Bazzania trilobata*.[8] Heptitol esters of lignans have also been isolated from the liverwort, *Lepicolea ochroleuca*.[9] It seems that their structures are closely related from the biogenetic point of view. A continuous study of their biosynthesis will provide not only conclusive proof for the structure of **1**, but also their biogenetic relationship.

Compound **2**a gave the $[M-H]^-$ ion peak at *m/z* 295.04539 in the HR-FAB mass spectrum, corresponding to a molecular formula of $C_{13}H_{11}O_8$. Its UV spectrum showed maxima at 220, 245 and 330 nm. The ^{1}H- and ^{13}C-NMR data for **2a** were identical with the authentic data[18–20] for (2′*S*)-phaselic acid. However, the optical rotation of **2a** ($-37.7°$, in H_2O) is opposite to that of the authentic data ($+31.5°$, in H_2O).[20]

Compound **3** was identified as (−)-epiphyllic acid by a direct comparison with authentic data[2] (^{1}H- and ^{13}C-NMR spectra, FABMS and $[\alpha]_D$). Compound **4** gave the $[M-H]^-$ and $[M+Na-2H]^-$ ion peaks at *m/z* 473 and 495 in the negative-FAB mass spectrum, corresponding to a molecular weight of 474. The ^{1}H-NMR data for **4** are similar to those of **3**, except for new signals at δ_H 2.90 (2H, *d*, $J=6.3$ Hz) and 5.43 (1H, *t*, $J=6.3$ Hz). The ^{13}C-NMR data for **4** also showed new signals at δ_C 37.1 (CH_2), 70.2 (CH), 173.0 (C) and 173.8 (C). These observations together with the MS data indicate that **4** was a malic acid ester of **3**. Due to the low-field shift of H-2″ (δ_H 5.43) of the malic acid moiety, an ester bond between the alcoholic function of the malic acid and the carboxylic atom of the lignan was assumed. Long range coupling between H-2″ and C-9 in the COLOC spectrum indicated that the structure of **4** was 9,2″-epiphylloyl-malic acid. 3-Carboxy-6,7-dihydroxy-1-(3′,4′-dihydroxyphenyl)-naphthalene-9,2″-*O*-malic acid ester has recently been isolated from the



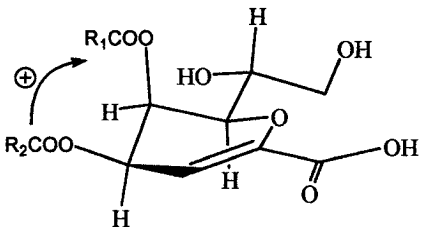


Fig. 4. CD Spectrum of Compound **1**.

liverwort, *Chiloscyphus polyanthos*.[21] However, the stereochemistry of the malic acid moiety has not been elucidated.

Hydroxycinnamic acid esters of glyceric, threonic, tartaric and malic acid are widely distributed in vascular plants.[22] In particular, (2′*S*)-phaselic acid is *trans*-caffeoyl-L-malic acid which has been isolated from the kidney bean (*Phaseolus vulgaris*),[23] red clover (*T. pratense*),[18] small radish (*Raphanus sativus*),[19] *Chelidonium majus*[20] and *Trifolium subterraneum*.[24] (2′*S*)-Phaselic acid has also been detected in some vegetables.[25] L-Malic acid has been considered to be the only naturally occurring form, and *trans*-caffeoyl-D-malic acid has been isolated from *Fumaria officinalis*.[22] Interest in its diversity prompted us to determine the configurations of the malic acids in **2a** and **4**. To compare with an authentic sample, *trans*-caffeoyl-L-malic acid (**2b**) was isolated from *T. pratense*. Malic acids obtained from alkaline hydrolysis of **2a**, **2b** and **4** were esterified with diazomethane and *p*-bromobenzoylated to afford **5** s. Chiral HPLC analyses of **5** s indicated that **2a** was (+)-*trans*-caffeoyl-D-malic acid and that **4** was (−)-9,2″-epiphylloyl-L-malic acid, respectively (Table 3). The distribution of both enantiomers of malic acid in liverworts seems to be dependent on biological diversity. Further feeding experiment of labeled fumaric acid and maleic acid may reveal their biosynthetic relationship.

To characterize compound **1**, its *in vitro* antioxidative activity was measured by the erythrocyte membrane ghost system.[17] The activity of **1** was similar to that of α-tocopherol as shown in Table 4. Subulatin (**1**) might play a role in the detoxification of oxygen generated by photooxidation in the liverwort.

Table 3. Enantiomeric Compositions of **5** s Derived from **2a**, **2b** and **4**

Compound (origin)	**5** s
2a (*Jungermannia subulata*)	D-(+) (91.2% e.e.)
4 (*Lophocolea heterophylla*)	L-(−) (100% e.e.)
2b (*Trifolium pratense*)	L-(−) (100% e.e.)

e.e.: enantiomeric excess.

Table 4. Antioxidative Assay of Compound **1** by the Erythrocyte Membrane Ghost System

Sample (mM)	Lipid peroxidation (%)				
	0	0.01	0.1	1	10
α-Tocopherol	100	95	91	77	61
Compound **1**	100	95	96	87	45

The values obtained without an antioxidant were taken as representing 100% lipid peroxidation.

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