Phytochemistry, Vol. 26, No. 12, pp. 3181–3185, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00

LICHEN ACIDS, PLANT GROWTH INHIBITORS FROM *USNEA LONGISSIMA*

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(*Revised received* 12 *April* 1987)

Key Word Index—*Usnea longissima*; depside; β-orcinol; β-orcinolcarboxylic acid; plant growth inhibitor; allelopathy.

Abstract—Eight components, depsides and orcinol derivatives which exhibit growth-inhibitory activity against lettuce seedlings were isolated from *Usnea longissima* and identified from their physicochemical data.

INTRODUCTION

Since Molisch defined the term 'allelopathy' in 1937 [1], many scientists have been concerned with the exploration and exploitation of allelopathic chemicals [2–15]. We are interested in the biochemical interactions between lichen and other higher plants [6, 17].

It is said that the vigorous propagation of lichens such as *Usnea* and *Parmelia* species on trees gives rise to inhibition of growth and finally to their death.

U. longissima, which is a filamentous type of lichen, adheres extensively to pine trees and grows in foggy areas, especially Tokachi, in the eastern part of Hokkaido and Hidaka, central Hokkaido. Until now barbatic acid (**1**), (+) usnic acid [18], diffractaic acid (**2**), protocetraric acid [19], evernic acid (**3**) [20], atranorin (**4**), fumarprotocetraric acid and salazinic acid [21], have been previously isolated from, *U. longissima*, but the biological activity of them on the growth of higher plants has not been clarified [22].

This paper deals with the isolation and identification of compounds in *U. longissima* which exhibit growth inhibitory activity against higher plants, and also the quantitative analysis of major components using reverse-phase HPLC in connection with allelopathic evaluations.

RESULTS AND DISCUSSION

Plant growth inhibitors

Full details of the isolation and physicochemical properties, ^{1}H NMR, ^{13}C NMR, mass and IR spectra of depside, β-orcinol and dibenzofuran type of compounds from *U. longissima* are given in the Experimental. Benzene was found to be the most efficient extraction solvent.

Lichens were collected in Furano, central Hokkaido, and extracted with benzene. After standing, yellow crystals appeared in the solution. These were identified as (+)usnic acid from spectral data [23, 24], although growth inhibitory activity against lettuce seedlings was very low even at high concentration. The filtrate after removal of usnic acid was concentrated and fractionated by silica gel CC. Fractionation was monitored by growth inhibitory activity. Each active fraction was purified by further CC and reverse phase HPLC. As a result, nine active compounds and a related inactive compound were isolated. Each compound was identified by the interpretation of their physicochemical properties (see Experimental).

Eight active compounds were identified as 4-*O*-methylorsellinic acid (**5**), barbatic acid (**1**), evernic acid (**3**), diffractaic acid (**2**), β-orcinolcarboxylic acid (**6**), 4-*O*-demethylbarbatic acid (**7**) orsellinic acid (**8**) and lecanolic acid (**9**)(F-9). One of the related inactive compounds was identified as 4-*O*-methylorsellinic acid ethyl ester (**10**).

One unknown compound having growth inhibitory activity was analysed by spectroscopic methods. The field desorption mass spectrum of the compound showed a $[M]^+$ at *m/z* 390 (This ion was not detected in the EI spectrum). The ^{1}H NMR was similar to that of diffractaic acid (**2**), except for the appearance of a signal at δ4.66 ppm (2H) instead of the signal at δ2.13 ppm (3H, Me bonded to aromatic ring). This observation indicated that one proton of the methyl group bonded to the aromatic ring was substituted by a hetero atom, since the signal of methylene protons was shifted to low field at δ4.66 ppm. The EI mass spectrum showed a base peak at *m/z* 209 (high resolution 209.0774 for $C_{11}H_{13}O_4$). This fragment ion (**11**), a characteristic fragmentation of ester ($R-C\equiv O^+$), comes from the A ring. Therefore, the unknown compound was identified as 3-α-hydroxydiffractaic acid (**12**) a new natural product. The new compound is comparable to 3-α-hydroxybarbatic acid (**13**) which has been found in *Xanthoparmelia moctezumensis* [25] and methyl 3-α-hydroxy-4-*O*-demethylbarbatate (**14**) in *Oropogon loxensis* [26]. However, the oxidation type at C-3 (hydroxymethyl group) is unique and the biosynthesis of **12** is very interesting. The new compound (**12**) may be formed by esterification of **15** as A ring and **6** as B ring followed by methylation of the hydroxy group, or/and by oxidation at the C-3 methyl group of diffractaic acid (**2**).

Growth inhibitory activity

The growth inhibitory-activity of some compounds isolated from *U. longissima* on lettuce seedling is shown in Figs 1–3. Of the depsides, 4-*O*-demethylbarbatic acid (**7**) exhibited the highest activity. The number of methyl groups bonded to the benzene rings and to the phenolic oxygen are important in terms of activity.

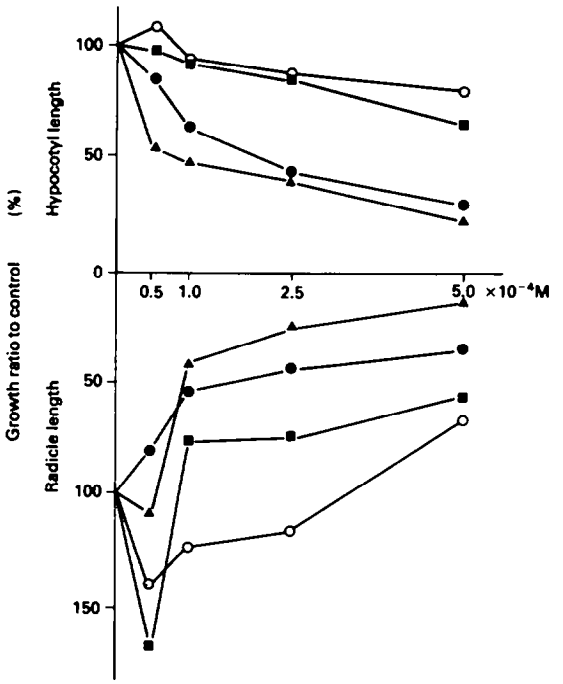

Fig. 1. Growth inhibitory activities of some depsides. O, Barbatic acid; ●, diffractaic acid; ■, evernic acid; ▲, 4-*O*-demethylbarbatic acid.

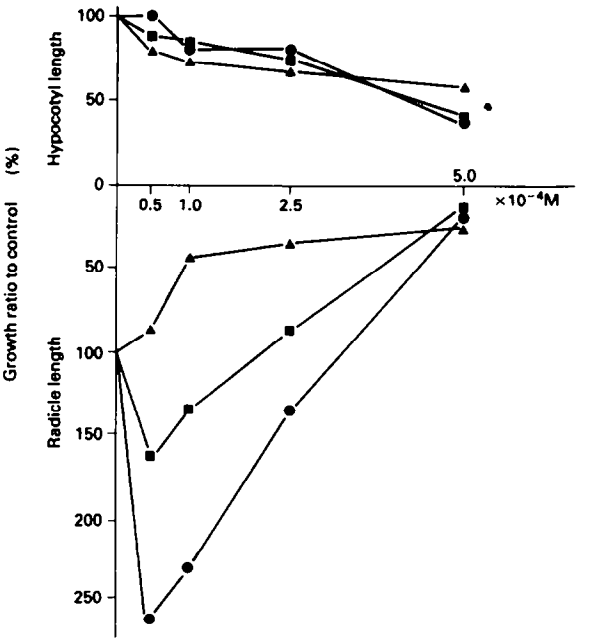

Fig. 3. Growth inhibitory activities of β-orcinolcarboxylic acid derivatives. ▲, 4-*O*-Methyl-β-orcinolcarboxylic acid; ●, 2,4-*O*-dimethyl-β-orcinolcarboxylic acid; ■ β-orcinolcarboxylic acid.

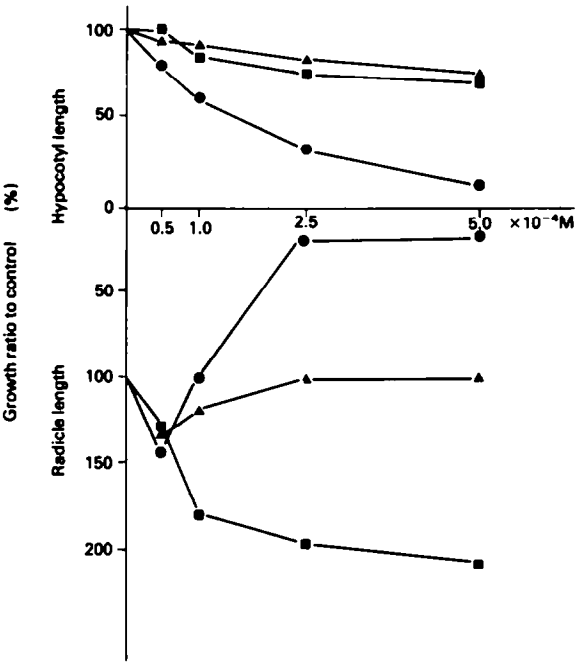

Fig. 2. Growth inhibitory activities of orsellinic acid derivatives. ▲, Orsellinic acid; ●, 4-*O*-methyl orsellinic acid; ■, 4-*O*-methyl orsellinic acid ethyl ester.

To investigate the relationship between structure and activity, the depsides **1**, **2** and **3** were hydrolysed by concentrated sulphuric acid (Fig. 4). The growth inhibitory activities of the hydrolysis products are shown in Figs 2 and 3. The structural difference is the number of hydroxy and methoxy groups bonded to the benzene ring. The compounds inhibit the growth of radicle.

β-Orcinol type depsides showed higher activity than orcinol type depsides. The nature of the substituents (hydroxy or methoxy) at the 2- and 4-positions affected the solubility in acetone and their biological activity. 3-α-Hydroxydiffractaic acid (**12**) exhibited 90% radicle length and 60% hypocotyl length inhibitory activity to control at 4.0×10^{-4} M.

To fully elucidate the relationship between structure and activity, we need to investigate the effect of oxidation on the C-1 substitution unit at the 3-position and some other classes of hydroxy and alkylated hydroxy groups.

Quantitative analysis and allelopathic evaluation

The amount of depsides **1**–**3** and **7** were determined by reverse phase HPLC to evaluate the possibility as allelochemics from an ecological viewpoint. Benzoic acid was used as an internal standard. The crude extract was analysed after removal of usnic acid by recrystallization. Usnic acid comprises 1.5% of the dry weight, but it has little activity on lettuce seedlings even at high concentrations. Other components could be separated on Unisil C-18 (Gasukuro Kogyo Ltd.) with UV detector at 274 nm using methanol–water–acetic acid (90 : 10 : 1). The amount of each component was estimated from the HPLC data (Table 1). Although barbatic acid (**1**) does not have activity as high as the other compounds, it occurs in *U. longissima* in relatively large amounts (2.0% of dry wt). Thus it would appear that barbatic acid contributes to the biological activity of the crude extract and is significant as an allelochemical.

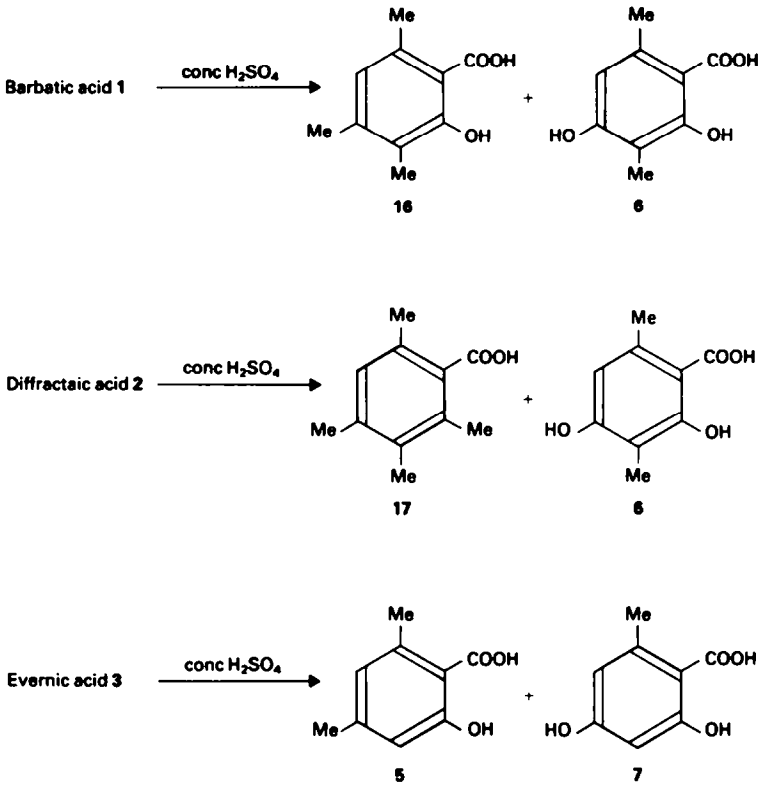

Fig. 4. Hydrolysis of depsides.

Table 1. The amount of lichen acid constituents in *Usnea longissima*

Compound	Concentration (%) by HPLC
Usnic acid	2.4*
4-*O*-Demethylbarbatic acid	0.2
Evernic acid	0.7
Diffractaic acid	1.4
Barbatic acid	2.0

*g/dry weight.

It is difficult to distinguish among subspecies of *U. longissima* from their external form and it is not clear which one is the standard species. However, subspecies of *U. longissima* have been recently distinguished and classified by the quantitative comparison of major components such as barbatic acid (**1**), diffractaic acid (**2**) and evernic acid (**3**).

EXPERIMENTAL

Plant material. Usnea longissima was collected at an altitude of *ca* 1000 m in the Tokyo University Forest in Hokkaido, Furano-079-15, Japan. It adheres to *Taxus* and *Picea* species.

Isolation of active compounds. Dry *U. longissima* (557 g) was soaked in 30 l of C_6H_6 at room temp. for *ca* three weeks. The C_6H_6 extract (38.7 g) was evapd and then redissolved in hot C_6H_6. While standing, the crude extract gave yellow crystals. (13.7 g). The yellow compound was identified as usnic acid from its spectral data. The filtrate was concd and subjected to silica gel CC using gradient elution with $CHCl_3$–EtOAc to give 14 fractions. According to bioassay using lettuce seedlings, active fractions 4 (0.82 g), 5 (1.77 g), 6 (8.26 g), 7 (3.61 g), 8 (0.39 g) and 9 (0.32 g) were obtained.

Orcinol, *β*-orcinol derivatives and *para* depsides were isolated by further silica gel CC and HPLC (Unisil C-18, 7.6 × 30 cm). 4-*O*-Methylorsellinic acid (**5**) was obtained from F-4, barbatic acid (**1**) from F-4 and 5, diffractaic acid (**2**) and evernic acid (**3**) from F-7-9, *β*-orcinolcarboxylic acid (**6**) and 4-*O*-demethylbarbatic acid (**7**) from F-7, orsellinic acid (**8**) and lecanolic acid (**9**) and 3-*α*-hydroxydiffractaic acid (**12**) from F-9.

General. Mp are uncorr. NMR spectra were recorded at 270 MHz.

Bioassay. Samples were dissolved in 125 *μ*l $(Me)_2CO$ and added to 15 ml 0.35% agar soln. After the agar soln. had solidified, 15 seedlings were put on the agar in a deep Petri dish (60 × 60 mm diam.) and incubated at 22° for 7 days in 14 hr light and 10 hr dark. Hypocotyl and radicle lengths were measured and compared with control.

4-O-Methylorsellinic acid; 2-hydroxy-4-methoxy-6-methylbenzoic acid (**6**) [27]. Mp 167 ~ 169° (from $(Me)_2CO$). IR ν_{max}^{KBr} cm^{-1}: 3600, 3300, 3000, 1620. FDMS *m/z*; 182 $[M]^+$ (100). High resolution EIMS *m/z*; 182.0555 (calcd for $C_9H_{10}O_4$, 182.0579). EIMS *m/z* (%); 182 $[M]^+$ (43), 164 $[M-H_2O]^+$ (100). 138 $[M-CO_2]^+$ (11), 136 (61), 109 (13). ^1H NMR (270 MHz, $CDCl_3$, TMS); *δ*2.57 (3H, *s*, 4-OMe), 6.33 (1H, *d*, *J* = 2.54 Hz, H-3), 6.35 (1H, *d*, *J* = 2.54 Hz, H-5), 11.56 (1H, *s*, 2-OH). ^{13}C NMR (270 MHz, $CDCl_3$, TMS); *δ*24.28 (*q*, C-9), 55.72

(q, C-8), 99.46 (d, C-3), 105.50 (s, C-1), 111.43 (d, C-5), 144.46 (s, C-6), 165.12 (s, C-2), 167.17 (s, C-4), 174.13 (s, C-7).

Barbatic acid; 2-*hydroxy*-2-*methoxy*-3,6-*dimethylbenzoic acid* 4'-*carboxy*-3'-*hydroxy*-2',5'-*methylphenyl ester* (**1**) [28]. Mp 181 ~ 183° (from EtOAc). IR ν_{max}^{KBr} cm^{-1}: 3220, 1665, 1630. FDMS *m/z* (%); 361 $[M+H]^+$ (27), 360 $[M]^+$ (100). High resolution EIMS *m/z*; 360.1198 (calcd for $C_{19}H_{20}O_7$, 360.1209). EIMS *m/z* (%); 360 $[M]^+$ (0.4), 196 (15), 182 (13), 179 (100), 178 (19), 164 (12), 136 (31). ^{1}H NMR [270 MHz, $(CD_3)_2CO$, TMS]; δ2.05 (3H, *s*, 3-Me), 2.06 (3H, *s*, 2'-Me), 2.61 (3H, *s*, 5'-Me), 3.95 (3H, *s*, 4-OMe), 6.62 (1H, *s*, H-5), 6.72 (1H, *s*, 6'-H), 11.46 (1H, *s*, 3'-OH). ^{13}C NMR (270 MHz, $(CD_3)_2CO$, TMS); δ8.5 (*q*, C-8), 9.9 (*q*, C-7'), 24.3 (*q*, C-10), 25.4 (*q*, C-9'), 56.7 (*q*, C-9), 105.8 (*s*, C-3), 108.1 (*d*, C-5), 111.0 (*s*, C-1), 111.9 (*s*, C-2'), 117.8 (*d*, C-6' and *s*, C-4'), 141.7 (*s*, C-6), 142.4 (*s*, C-5'), 154.2 (*s*, C-2), 163.8 (*s*, C-1'), 164.0 (*s*, C-3'), 164.7 (*s*, C-4), 171.4 (*s*, C-7), 174.8 (*s*, C-8').

Evernic acid; 2-*hydroxy*-4-*methoxy*-6-*methylbenzoic acid* 4'-*carboxy*-3'-*hydroxy*-5'-*methylphenyl ester* (**3**) [29, 30]. Mp 169 171° (from MeOH). IR ν_{max}^{KBr} cm^{-1}: 3450, 3050, 1660, 1610, FDMS *m/z* (%); 333 $[M+H]^+$ (24), 332 $[M]^+$ (100). High resolution EIMS *m/z*; 332.0871 (calcd for $C_{17}H_{18}O_7$, 334.1052). EIMS *m/z* (%); 332 $[M]^+$ (0.4), 168 (34), 165 (75), 164 (86), 136 (44). ^{1}H NMR [270 MHz, $(CD_3)_2CO$, TMS]; δ2.64 (3H, *s*, 6-Me), 2.65 (3H, *s*, 5'-Me), 3.87 (3H, *s*, 4-OMe), 6.41 (1H, *d*, J = 2.47 Hz, 5-H), 6.47 (1H, *d*, J = 2.44 Hz, 3-H), 6.74 (1H, *d*, J = 2.47 Hz, 2'-H), 6.82 (1H, *d*, J = 2.47 Hz, 6'-H), 11.17 (1H, *s*, 3'-COOH). ^{13}C NMR [270 MHz, $(CD_3)_2CO$, TMS]; δ24.4 (*q*, C-9), 24.8 (*q*, C-8'), 56.4 (*q*, C-8), 100.3 (*d*, C-3), 106.2 (*s*, C-1), 110.0 (*d*, C-2'), 111.8 (*s*, C-4'), 112.6 (*d*, C-5), 117.8 (*d*, C-6'), 144.8 (*s*, C-6), 145.2 (*s*, C-5'), 155.7 (*s*, C-3'), 166.0 and 166.3 (each *s*, C-2 and C-1'), 167.3 (*s*, C-4), 170.7 (*s*, C-7), 174.0 (*s*, C-7').

Diffractaic acid; 2,4-*dihydroxy*-3,6-*dimethylbenzoic acid* 4'-*carboxy*-3'-*hydroxy*-2',5'-*dimethylphenyl ester* (**2**) [28]. Mp 190 ~ 194° [from $(Me)_2CO$]. IR ν_{max}^{KBr} cm^{-1}: 3450, 3050, 1740, 1640, FDMS *m/z* (%); 375 $[M+H]^+$ (25), 374 $[M]^+$ (100). High resolution EIMS *m/z*; 374.1368 (calcd for $C_{20}H_{22}O_7$, 374.13656). EIMS *m/z* (%); 374 $[M]^+$ (0.4), 210 (0.5), 194 (100), 193 (3), 182 (4), 178 (15), 165 (5), 164 (4), 136 (2). ^{1}H NMR [270 MHz, $(CD_3)_2CO$, TMS]; δ2.13 (3H, *s*, 3-Me), 2.14 (3H, *s*, 2'-Me), 2.45 (3H, *s*, 6-Me), 2.62 (3H, *s*, 5'-Me), 3.84 (3H, *s*, 4-OMe), 6.68 (1H, *s*, 5-H), 6.76 (1H, *s*, 6'-H), 11.69 (1H, *s*, 2'-OH).

β-Orcinolcarboxylic acid; 2,4-*dihydroxy*-3,6-*dimethylbenzoic acid* (**6**) [31]. IR ν_{max}^{KBr} cm^{-1}: 3400, 1635, FDMS *m/z* (%); 183 $(M+H]^+$ (22), 182 $[M]^+$ (100). High resolution EIMS *m/z*; 182.0576 (calcd for $C_9H_{10}O_4$, 182.0579). EIMS *m/z* (%); 182 $[M]^+$ (17), 164 $[M-H_2O]^+$ (30), 138 $[M-CO_2]^+$ (100), 136 $[M-CO_2-H_2O]^+$ (43), 123 (41), 109 (14). ^{1}H NMR [270 MHz, $(CD_3)_2CO$, TMS]; δ2.03 (3H, *s*, 3-Me), 2.49 (3H, *s*, 6-Me), 6.34 (1H, *s*, 5-H).

4-O-*Demethylbarbatic acid*; 2,4-*dihydroxy*-3,6-*dimethylbenzoic acid* 4'-*carboxyl*-3'-*hydroxy*-2',5'-*dimethylphenyl ester* (**7**) [32]. Mp 174 ~ 175° (from Me_2CO). IR ν_{max}^{KBr} cm^{-1}: 3400, 3050, 1655, 1635. FDMS *m/z* (%); 347 $[M+H]^+$ (33), 346 $[M]^+$ (100). High resolution EIMS *m/z*; 346.1074 (calcd for $C_{18}H_{18}O_7$, 346.10524). EIMS *m/z* (%); 346 $[M]^+$ (0.2), 182 (39), 165 (68), 164 (72), 138 (19), 37 (20), 36 (100). ^{1}H NMR [270 MHz, $(CD_3)_2CO$, TMS]; δ2.07 (3H, *s* 3-Me), 2.09 (3H, *s*, 2'-Me), 2.60 (3H, *s*, 6-Me), 2.61 (3H, *s*, 5'Me), 6.46 (1H, *s*, 5-H), 6.69 (1H, *s*, 6'-H).

Orsellinic acid; 2,4-*dihydroxy*-6-*methylbenzoic acid* (**8**) [33]. IR ν_{max}^{KBr} cm^{-1}: 3530, 3440, 1605. FDMS *m/z* (%); 169 $[M+H]^+$ (24), 168 $[M]^+$ (100). High resolution EIMS *m/z*; 168.0414 (calcd for $C_8H_8O_4$, 168.04224). EIMS *m/z* (%); 168 $[M]^+$ (52), 151 $[M-OH]^+$ (100), 124 $[M-CO_2]^+$ (79), 122 $[M-H_2O-CO]^+$ (56). ^{1}H NMR [270 MHz, $(CD_3)_2CO$, TMS]; δ2.52 (3H, *s*, 6-Me), 6.23 (1H, *d* J = 2.56 Hz, 3-H), 6.29 (1H, *d*, J = 2.56 Hz, 5-H).

Lecanolic acid; 2,4-*dihydroxy*-6-*methylbenzoic acid* 4'-*carboxy*-3'-*hydroxy*-5'-*methylphenyl ester* (**9**) [29]. Mp 174 ~ 175° (from Me_2CO). IR ν_{max}^{KBr} cm^{-1}: 3550, 3450, 3110, 1665, 1650, FDMS *m/z* (%); 319 $[M+H]^+$ (23), 318 $[M]^+$ (100). EIMS *m/z* (%); 167 (20), 149 (100). ^{1}H NMR [270 MHz, $(CD_3)_2CO$, TMS]; δ2.51 (3H, *s*, 6-Me), 2.65 (3H, *s*, 5'-Me), 6.31 (1H, *d*, J = 2.45 Hz, 3-H), 6.40 (1H, *d*, J = 2.44 Hz, 2'-H), 6.72 (1H, *d*, J = 2.45 Hz, 5-H), 6.76 (1H, *d*, J = 2.44 Hz, 6'-H).

3-α-*Hydroxydiffractaic acid*; 3-*hydroxymethyl*-2,4-*dimethoxy*-6-*methylbenzoic acid* 4'-*carboxy*-3'-*hydroxy*-2',5'-*dimethylphenyl ester* (**12**). Mp 156 ~ 158° (from EtOH). IR ν_{max}^{KBr} cm^{-1}: 3450, 1735, 1655. FDMS *m/z* (%); 391 $[M+H]^+$ (41), 390 $[M]^+$ (100). High resolution EIMS *m/z*: 209.0774 (calcd for $C_{11}H_{13}O_4$, 209.08130). EIMS *m/z* (%); 209 (100). ^{1}H NMR (270 MHz, $(CD_3)_2CO$, TMS); δ2.13 (3H, *s*, 2'-Me), 2.47 (3H, *s*, 5'-Me), 2.63 (3H, *s*, 6-Me), 3.91 (3H, *s*, 2-OMe), 3.97 (3H, *s*, 4-OMe), 4.66 (2H, *s*, 3-CH_2OH), 6.65 (1H, *s*, 5-H), 6.82 (1H, *s*, 6'-H).

4-O-*Methylorsellinic acid ethyl ester*; *ethyl* 2-*hydroxy*-4-*methoxy*-6-*methylbenzoate* (**10**). IR ν_{max}^{KBr} cm^{-1}: 3400, 1640, FIMS *m/z*; 211 $[M+H]^+$, 210 $[M]^+$. High resolution EIMS *m/z*; 210.0908 (calcd for $C_{11}H_{14}O_4$, 210.08922). EIMS *m/z* (%); 210 $[M]^+$ (22), 165 $[M-EtO]^+$ (28), 164 $[M-EtOH]^+$ (100), 136 $[M-EtOH-CO]^+$ (36). ^{1}H NMR (270 MHz, $CDCl_3$, TMS); δ1.41 (3H, *t*, J = 7.32 Hz, 2'-Me), 2.51 (3H, *s*, 6-Me), 3.80 (3H, *s*, 4-OMe), 4.39 (2H, *q*, J = 7.32 Hz, 1'-CH_2), 6.28 (1H, *d*, J = 2.44 Hz, 3-H), 6.32 (1H, *d*, J = 2.44 Hz, 5-H), 11.85 (1H, *s*, 2-OH).

Hydrolysis [34]. Depsides (0.1 mmol) were dissolved in 1 ml conc H_2SO_4 at 0° and allowed to stand for 10 min. The soln changed to yellow in colour. Ice H_2O (5 ml) was poured into the soln and the white amorphous ppt. extd × 3 with 10 ml Et_2O. The Et_2O layer was washed with brine and dried ($MgSO_4$). The mixt. of hydrolysis products from the A and B rings of the depsides were sepd by prep. TLC using hexane-*iso* PrOH–HOAc (8 : 1 : 0.9).

4-O-*Methyl*-β-*orcinolcarboxylic acid as A ring of* **1**; 2-*hydroxy*-4-*methoxy*-3,6-*dimethylbenzoic acid* (**16**). IR ν_{max}^{KBr} cm^{-1}: 3400, 3050, 1620. FDMS *m/z* (%); 197 $[M+H]^+$ (25), 196 $[M]^+$ (100). High resolution EIMS *m/z*; 196.0757 (calcd for $C_{10}H_{12}O_4$, 196.07356). EIMS *m/z* (%); 196 $[M]^+$ (8), 178 $[M-H_2O]^+$ (10), 152 $[M-CO_2O]^+$ (19), 150 $[M-H_2O-CO]^+$ (15), 138 (27). ^{1}H NMR (270 MHz, $CDCl_3$, TMS); δ2.00 (3H, *s*, 3-Me), 2.59 (3H, *s*, 6-Me), 3.89 (3H, *s*, 4-OMe), 6.48 (1H, *s*, 5-H).

2,4-O-*Dimethyl*-β-*orcinolcarboxylic acid as A ring of* **2**; 2,4-*dimethoxy*-3,6-*dimethylbenzoic acid* (**17**). IR ν_{max}^{KBr} cm^{-1}: 3050, 1690. FDMS *m/z* (%); 211 $[M+H]^+$ (16), 210 $[M]^+$ (100). High resolution EIMS *m/z*; 210.0856 (calcd for $C_{11}H_{14}O_4$, 210.0892). EIMS *m/z* (%); 210 $[M]^+$ (71), 193 $[M-H_2O]^+$ (49), 177 (30), 163 (29), 150 (11), 149 (42). ^{1}H NMR (270 MHz, $CDCl_3$, TMS); δ2.14 (3H, *s*, 2-OMe), 2.59 (3H, *s*, 6-Me), 3.84 (3H, *s*, 4-OMe), 3.87 (3H, *s*, 2-OMe), 6.58 (1H, *s*, 5-H).

Acknowledgements—We are grateful fo Drs Ikuo Takahashi and Akio Kurahashi, the Tokyo University Forest in Hokkaido Furano 079-15, Japan, for collection of *U. longissima*.

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