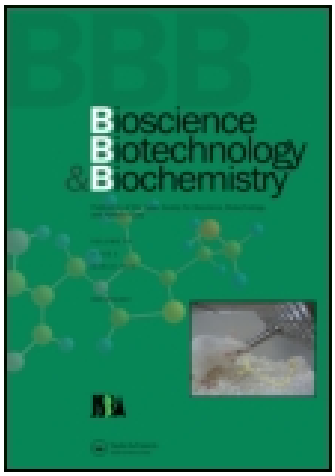

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Inhibiting Effects of Lunularic Acid Analogs on the Growth of Liverwort, Watercress, and Timothy Grass

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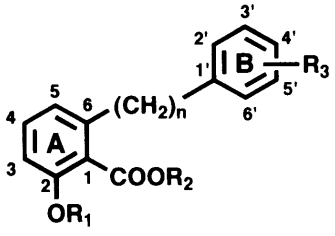
A number of analogs of lunularic acid varying in the number of methylene carbons between the two benzene rings and in the substituents on their rings were prepared, and their effects on the growth of liverwort gemmaling, watercress, and timothy grass were investigated. Almost all the analogs tested were more inhibitory than lunularic acid, and a correlation between the structure and activity was observed. The differences in the growth-inhibition activity of analogs between higher and lower plants are also discussed.

Key words: lunularic acid; *Marchantia polymorpha*; *Nasturtium officinale*; *Phleum pratense*; growth inhibition

Lunularic acid (2-hydroxy-6-[2-(4-hydroxyphenyl)ethyl]-benzoic acid; **1**) is a naturally occurring growth inhibitor of liverwort,[1] which was first isolated from *Lunularia cruciata* (L.) DUM.[2] Lunularic acid has also been reported to have growth-inhibition activity against higher plants, and the two hydroxyl groups on both benzene rings in its structure seemed to be important for this activity.[3,4] The preparation of some lunularic acid analogs, mainly substituted dihydrostilbene derivatives, and their effects on the growth of liverwort gemmaling and on the elongation of cress root have already been reported by Gorham.[5] However, he could find no clear correlation between their structures and biological activities. To find new information about the structure–activity relationship of lunularic acid analogs, this study was conducted to prepare analogs varying in the number of methylene carbons between the two benzene rings, and to optimize the substituents on the aromatic rings (see Fig.). We report the results of growth-regulation assays of these new lunularic acid analogs against liverwort (*Marchantia polymorpha* L.), dicotyledonous watercress (*Nasturtium officinale* R. Br.) and monocotyledonous timothy grass (*Phleum pratense* L.).

First, the effects of different numbers of methylene carbons (represented as *n* in the text and tables) between the A- and B-rings of lunularic acid (see Fig. and Table I) on the growth of the three plant species were investigated. As shown in Table I, the growth-inhibition activities of analogs **5**–**7** and of corresponding 4′-*O*-methyl derivatives **9**–**11** ($n=4$, 5, and 6) against gemmalings were markedly stronger than that of lunularic acid. Analogs **6** and **9** ($n=5$ and 4 possessing 4′-hydroxy- and 4′-methoxy-substituted B-rings, respectively) were the most inhibitory among the compounds possessing the same substituents. In contrast, the analogs with larger numbers of methylene carbons inhibited more markedly *N. officinale*, and when both compounds had the same number of methylene carbons, the 4′-*O*-substituted derivative was more inhibitory than the 4′-hydroxyl derivative (*e.g.*, **5** and **9**, **6** and **10**, and **7** and **11**). Although this correlation was not clear in the activities against *P. pratense*, **11** also appeared to be the most inhibitory among analogs **5**–**7** and **9**–**11**. However, additional modifications to the carboxyl and/or hydroxyl group on the A-ring resulted in a reduction in inhibitory activity, except for **22**, even in the analogs possessing a relatively large number of methylene carbons (**17**–**21**, **23**). Analogs **2**, **3**, and **4** ($n=0$, 1, and 3, respectively) exhibited no or very weak inhibition against the liverwort gemmalings and higher plants, *N. officinale* and *P. pratense*.

Next, analogs with different substituents on the B-ring were subjected to the growth-regulation assays. Following the method proposed by Topliss for optimizing the arylic substituents,[6] five analogs (**8**, **24**–**27**) were first subjected to the assays. The results are summarized in Table II. Against the liverwort gemmalings, **25** showed the strongest inhibition, the activities of **8**, **24**, and **26** being almost same. From these results, the potency order of analogs against liverwort gemmalings was **25** (4′-Cl) > **24** (3′,4′-Cl_2), **26** (4′-Me), **8** (4′-OMe) > **27** (H; no substituents on the B-ring). This result did not fit any of the potency orders shown in ref. 6 to deduce the parametric dependency of biological



n: 0 - 6
R_1: H, Me
R_2: H, Et
R_3: H, 4'-OH, OMe, OEt, OCH_2Ph, Cl, Me, 3',4'-Cl_2, 3'-CF_3-4'-Cl, 3',5'-Cl_2

Fig. General Structure of Lunularic Acid Analogs
(*cf.* lunularic acid (**1**) $n=2$, $R_1=R_2=H$, $R_3=4'$-OH).

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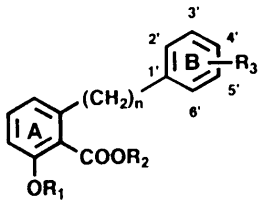
Table I. Growth-inhibition Activities of Lunularic Acid Analogs Differing in the Number of Methylene Carbons between the A- and B-Rings

Compd.	n[b]	Substituent[b]			Test plant[a]			
					Mp[c]		No[d]	Pp[d]
		R_1	R_2	R_3	2×10^{-10}	2×10^{-9}		
2	0	H	H	4′-OH	NT[e]	−	+	+
3	1	H	H	4′-OH	NT	−	+	−
1	2	H	H	4′-OH	NT	+	+ +	+ +
4	3	H	H	4′-OH	NT	−	+	−
5	4	H	H	4′-OH	NT	+ + + +	+ +	+ +
6	5	H	H	4′-OH	+ + + +	+ + + +	+ +	+ +
7	6	H	H	4′-OH	+	+ + + +	+ +	+
8	2	H	H	4′-OMe	+ +	+ + + +	+ +	+ +
9	4	H	H	4′-OMe	+ + +	+ + + +	+ + +	NT
10	5	H	H	4′-OMe	+ +	+ + + +	+ + +	+ +
11	6	H	H	4′-OMe	+	+ + + +	+ + + +	+ + + +
12	0	H	Et	4′-OMe	NT	NT	+	+ +
13	1	H	H	4′-OEt	NT	NT	+ +	+
14	2	Me	H	4′-OCH_2Ph	NT	NT	+ + +	+ +
15	2	Me	Et	4′-OCH_2Ph	NT	NT	−	−
16	3	Me	Et	4′-OMe	NT	NT	+	+[f]
17	4	H	Et	4′-OMe	NT	NT	+	+[f]
18	4	Me	Et	4′-OMe	NT	NT	+	+[f]
19	5	Me	H	4′-OMe	NT	NT	+ +	+ +
20	5	Me	Et	4′-OMe	NT	NT	−	+ +
21	6	H	Et	4′-OMe	NT	−	−	+
22	6	Me	H	4′-OMe	NT	NT	+ + +	+ +
23	6	Me	Et	4′-OMe	NT	NT	+	+[f]

[a] Mp, *Marchantia polymorpha*; No, *Nasturtium officinale*; Pp, *Phleum pratense*.

[b]



n: 0 6
R_1: H, Me
R_2: H, Et
R_3: 4′-OH, OMe, OEt, OCH_2Ph

[c] Relative inhibitory activity was evaluated by the increase in surface area of the test gemmaling compared to that of control (=100) and classified into five levels: + + + +, 0–25; + + +, 26–50; + +, 51–75; +, 76–100; −, >100. Figures under the plant name express the dosage of the test compound (mol/disc).

[d] Inhibitory activity was evaluated by the estimated range of IC_{90} (nM) and classified into five levels: + + + +, <0.01; + + +, 0.01–0.1; + +, 0.1–1.0; +, less inhibitory than 90% of the control at 1.0 mM; −, no inhibition at 1.0 mM.

[e] Not tested.

[f] Coleoptile growth was slightly inhibited, while root elongation (200–300% of the control) was observed at 1.0 mM.

activity relating to hydrophobic, electronic, and steric effects. So the Topliss' method was not applicable to optimize the substituents for stronger inhibitory activity against liverwort. Against the higher plants, *N. officinale* and *P. pratense*, the potency order of the first five analogs for growth inhibition was **24** (3′,4′-Cl_2)>**25** (4′-Cl)≥**26** (4′-Me)>**27** (H)>**8** (4′-OMe), and it seemed that the inhibitory activity against these higher plants was dependent on the parameter "$\pi+\sigma$", where π and σ represent the hydrophobic and electronic effects of the substituents, respectively. According to the instruction described by Topliss, two analogs with substituents 3′-CF_3-4′-Cl (**28**) and 3′, 5′-Cl_2 (**29**) on the B-ring, which were expected to be more potent against the higher plants, were selected. They were equally or more inhibitory compared with **24**, whilst they were less inhibitory than **25** against liverwort. So the potency order observed with liverwort did not agree well with the order against the higher plants.

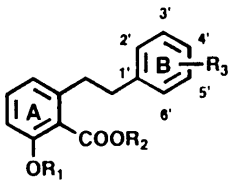
In regard to the A-ring, the presence of a free carboxyl group seems to have been important for growth-inhibition activity against both liverwort and the higher plants, because esterification of this carboxyl group resulted in reduced inhibitory activity (*e.g.*, **17** and **21** in Table I, and **43** in Table II). The hydroxyl group at position-2 of the A-ring seemed not to affect the inhibitory activity against the higher plants, because 2-*O*-methylated analogs **22** and **40** exhibited comparable activity to the corresponding compounds with a free hydroxyl group (**11** and **28**). Similar results were obtained with the other analogs with a free carboxyl group and 2-methoxyl group on the A-ring against the higher plants (**14** and **19** in Table I, and **30**, **32**, **34**, and **38** in Table II). These results further supported our supposition that the free carboxyl group was significant to the inhibitory activity, and that the role of the hydroxyl group was not as significant as that of the carboxyl group. In contrast to our supposition, however, the importance of the free hydroxyl group on the A-ring for growth-inhibition activity against rice seedlings has been pointed out by

Table II. Growth-inhibition Activities of Lunularic Acid Analogs with Various Substituent(s) on the B-Ring

Compd.	Substituent[b]			Test plant[a]			
				Mp[c]			
	R_1	R_2	R_3	2×10^{-10}	2×10^{-9}	No[d]	Pp[d]
1	H	H	4′-OH	NT[e]	+	+ +	+ +
24	H	H	3′,4′-Cl_2	+ +	+ + + +	+ + +	+ +
25	H	H	4′-Cl	+ + +	+ + + +	+ +	+ +
26	H	H	4′-Me	+ +	+ + + +	+ +	+ +
8	H	H	4′-OMe	+ +	+ + + +	+ +	+ +
27	H	H	H	+	+ + + +	+ +	+ +
28	H	H	3′-CF_3,4′-Cl	+	+ + + +	+ + +	+ + +
29	H	H	3′,5′-Cl_2	+ +	+ + + +	+ + +	+ + +
30	Me	H	3′,4′-Cl_2	NT	NT	+ +	+ +
31	Me	Et	3′,4′-Cl_2	NT	NT	+	+
32	Me	H	4′-Cl	NT	NT	+ +	+ +
33	Me	Et	4′-Cl	NT	NT	+	+
34	Me	H	4′-Me	NT	NT	+ +	+ +
35	Me	Et	4′-Me	NT	NT	+	+[f]
36	Me	H	4′-OMe	NT	NT	+	+[f]
37	Me	Et	4′-OMe	NT	NT	+	+[f]
38	Me	H	H	NT	NT	+ +	+ +
39	Me	Et	H	NT	NT	+	+[f]
40	Me	H	3′CF_3,4′-Cl	NT	NT	+ + +	+ + +
41	Me	Et	3′-CF_3,4′-Cl	NT	NT	+	+[f]
42	Me	H	3′,5′-Cl_2	NT	NT	+ + +	+
43	Me	Et	3′,5′-Cl_2	NT	+ +	+ +	+ +

[a,c,d,e,f] See the footnotes to Table I.

[b]



R_1: H, Me
R_2: H, Et
R_3: 3′,4′-Cl_2, 4′-Cl, 4′-Me, 4′-OMe, H, 3′-CF_3-4′-Cl, 3′,5′-Cl_2

Hashimoto *et al.*,[4] and Gorham has previously reported that analogs with a methoxycarbonyl group on the A-ring were more potent than those with a free carboxyl group for the growth inhibition of liverwort gemmalings.[5]

In conclusion, it became apparent that the growth-inhibition activity was strongly affected by the distance between both benzene rings and by the physicochemical properties of aryl subtituents on the B-ring of lunularic acid analogs. However the requirements for the physicochemical characteristics of these moieties (*i.e.*, the methylene carbon chain and B-ring) for stronger activity were a little different between higher and lower plants. It seems that an optimum length of the methylene carbon chain between both benzene rings (presumably $n=4$ to 5) exists for the strongest activity against liverwort, while analogs with a longer chain ($n=5$ or 6) seem to have stronger activity against higher plants, especially against *N. officinale*, and methylation of the hydroxyl group on the B-ring tends to enhance the activity. The parametric dependency of the potency order with higher plants in relation to substituents of the B-ring was also significantly different from that with liverwort. Such a phenomenon presumably arose from differences in the structural and electronic properties of the interacting sites in the higher and lower plants.

Experimental

Syntheses of lunularic acid analogs. All analogs except **2**, **3**, **12**, and **13** were synthesized by following almost the same method as that adopted by Eicher *et al.* for the synthesis of lunularic acid.[7] A general procedure involved condensing 2-ethoxycarbonyl-3-methoxybenzyltriphenylphosphonium bromide and an appropriate aldehyde by the Wittig reaction. The resulting stilbene derivative was subjected to catalytic hydrogenation of the double bond over H_2/Pd C, and then the phenolic hydroxyl group and/or carboxyl group were respectively deprotected to give a designated analog. The synthesis of **2** was achieved by applying the method used for the synthesis of ethyl 2-methoxy-6-methylbenzoate.[8] Michael addition of ethyl 3-oxobutanoate to 4-methoxycinnamaldehyde was followed by cyclization catalyzed by gaseous HCl to give ethyl 6-(4-methoxyphenyl)-2-oxocyclohex-3-enecarboxylate. Aromatization of the 2-oxocyclohexene ring was achieved by halogenating the double bond with pyridinium bromide perbromide in acetic acid and then dehalogenating with K_2CO_3 in DMF. Finally, **2** was yielded by deprotecting the phenolic hydroxyl and carboxyl groups. Analog **3** was synthesized by condensing ethyl 2-hydroxy-6-bromomethylbenzoate (the demethylation product of ethyl 2-methoxy-6-bromomethylbenzoate with $AlCl_3$ in CH_2Cl_2) and phenyl ethyl ether with $AlCl_3$ in CS_2 to give a mixture of 2-hydroxy-6-(4-ethoxybenzyl)- and 2-hydroxy-6-(2-ethoxybenzyl)benzoic acid. After separating these isomers by repetitive SiO_2 column chromatography (*n*-hexane/toluene) and preparative TLC (*n*-hexane/$CHCl_3$), the desired 4-ethoxy isomer was deprotected to give **3**.

All compounds were identified on the basis of spectroscopic analyses (EI-MS and ^{1}H-NMR).

Growth-regulation assay of gemmaling. The assay with liverwort gemmalings was carried out according to the method described by

Gorham.[5] Thalli of *M. polymorpha* were collected on the campus of Hokkaido University and cultivated on rock wool moistened with Voth's No. 3 nutrient solution[9] at 25°C with a day length of 14 h under fluorescent light. Gemmae of *M. polymorpha* were collected from the gemma cup on to 90 mm-diameter filter paper and then surface-sterilized by soaking together with the filter paper in a 0.1% $KMnO_4$ solution for 10 min. After being rinsed thoroughly with H_2O, the gemmae were transferred to another sterilized 90 mm-diameter filter paper. Preceding the assay, the gemmalings were grown on filter paper moistened with double-strength Voth's No. 3 nutrient solution[9] placed in a Petri dish for 24 h at 21°C with a day length of 10 h under fluorescent light. Forty microliters of a methanolic stock solution containing an appropriate quantity of the test compound was loaded on to a sterilized 21 mm-diameter paper disc, and the solvent was removed under reduced pressure. As a control, the same amount of MeOH was used. Ten gemmalings precultivated as just described were placed on each loaded paper disc, and duplicate discs for each test compound were placed on a sterilized filter paper slip (25 × 50 mm). Each slip was moistened with 1 ml of the sterilized nutrient solution and placed in a sterilized Petri dish. The test gemmalings were cultivated for 80 h under the same conditions as those used for precultivation. The surface area of each gemmaling was measured by drawing its shape on squared paper under a microscope equipped with drawing apparatus (Olympus BH-2 and BH2-DA) and then counting the squares. The results of duplicate experiments were averaged.

Growth-regulation assay for N. officinale and P. pratense. The assay method was originally described by Stevens and Merrill,[10] seeds of *N. officinale* and *P. pratense* used being purchased from a commercial source. To 3 ml of molten agar (0.5%) in a test tube (12 mm i.d. × 105 mm) was added 25 μl of an acetone solution containing an appropriate amount of the test compound. The contents in the tube were mixed thoroughly, and the final concentrations of each test compound were adjusted to 0.01, 0.05, 0.1, 0.25, 0.5, and 1 mM, respectively. As a control, 25 μl of acetone was added to the agar medium. Ten seeds of *N. officinale* and *P. pratense* were sown on the medium and cultivated at 25°C with a day length of 14 h under fluorescent light. Five days after sowing, the lengths of the root and hypocotile (*N. officinale*) or coleoptile (*P. pratense*) were measured. The results of duplicate experiments were averaged.

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