Phytochemistry, Vol. 23, No. 8, pp. 1607–1609, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00

PRELUNULARIC ACID IN LIVERWORTS

YOSHIMOTO OHTA, SETSUKO ABE, HAJIME KOMURA and MASAKI KOBAYASHI

Suntory Institute for Bioorganic Research, Wakayamadai, Shimamoto-cho, Osaka 618, Japan

(*Received* 11 *October* 1983)

Key Word Index—*Marchantia polymorpha*; Hepaticae; suspension culture; prelunularic acid; 'pre-aromatic' intermediate; biogenesis.

Abstract—Prelunularic acid, the first example of an intermediate with a 'pre-aromatic' structure in the phenylpropanoid–polymalonate pathway, was isolated from suspension-cultured cells of *Marchantia polymorpha*. Its structure, including its absolute configuration, was assigned on the basis of spectral properties, direct conversion into lunularic acid, and CD measurements on the bis(*p*-dimethylaminobenzoate) of the methyl ester. Prelunularic acid was also detected in several liverworts of Marchantiales and Jungermanniales, and appears to be the immediate precursor of lunularic acid instead of the previously postulated hydrangenol or hydrangeic acid.

INTRODUCTION

Lunularic acid (LNA, **1**), a naturally-occurring dihydrostilbene carboxylic acid, was isolated from a liverwort, *Lunularia cruciata* of Israel strain, as a dormancy factor [1], and was proved to be identical to the growth regulator previously found in another liverwort, *Marchantia polymorpha* [2]. Since then, LNA has been detected in most liverworts examined so far [3, 4].

It has been reported that an increased amount of LNA could be extracted from liverworts when methanol containing hydrochloric acid instead of methanol itself was used as the solvent [3]. During the course of our studies on the formation of LNA in suspension-cultured cells of *M. polymorpha*, we also noticed that the extraction of the cells under acidic conditions resulted in a better yield of LNA [5]. After careful examination of the methanol extract, we discovered the presence of a labile component that gives LNA upon treatment with acid or alkali [6]. In this paper we describe the isolation and structure determination of this labile compound, prelunularic acid (preLNA), together with its detection in some intact liverworts.

RESULTS AND DISCUSSION

The cells of *M. polymorpha* cultured for 14 days in a modified Murashige–Skoog medium were extracted with 90% methanol. The aqueous fraction obtained by evaporation of methanol followed by extraction with ether was chromatographed on a Sephadex LH-20 column using distilled water. A small portion of each chromatographic fraction was treated with dilute sulphuric acid, and the fractions that gave LNA were combined for further purification through successive chromatography on a Waters Sep-Pak C_{18} cartridge, cellulose and Sephadex LH-20 columns. Care was taken to avoid exposure of the sample to acidic or basic conditions and to maintain the temperature below 35° in handling the fractions in each step. Approximately 2 mg of a non-crystalline compound, which we name prelunularic acid (preLNA, **2**), was obtained from 80 g of the fresh cultured cells. An attempt to recrystallize this failed because of its easy conversion into LNA.

The IR spectrum of preLNA showed the presence of hydroxyl (3300), aromatic ring (1600, 1514, 830), carboxylate (1573, 1402) and enone groups (1652, 1610 cm^{-1}). Since the isolation processes were carried out in water, the nature of the counter-ion of the carboxylate is not clear. Brief treatment of an ethereal solution of preLNA in free acid form with diazomethane gave a methyl ester (ν_{max} 1735 cm^{-1}) and the appearance of a new 3H singlet at δ 3.78. The high resolution mass spectrum of the methyl ester showed a molecular formula of $C_{16}H_{18}O_5$ indicating preLNA to be a hydrated derivative of LNA. The UV spectra of preLNA in water at pH 12 showed time-dependent changes, and the final spectrum after 3 hr was identical to that of LNA at the same pH (Fig. 1). The appearance of a clear set of isosbestic points at 247, 284 and 307 nm indicated that preLNA was directly converted into LNA under basic conditions ($T_{1/2}$ = *ca* 45 min at room temperature). Under acidic conditions, similar changes in the UV spectra were also observed but at a much slower rate ($T_{1/2}$ = *ca* 20 min in 0.5 M H_2SO_4 at 65°) (data not shown). The ^1H NMR spectrum of preLNA revealed the presence of a *p*-substituted benzene ring (δ 6.86 and 7.21, each 2H, *d*) and a moiety of $CH_2CH(O-)CH_2$ (1H at δ 4.26 and four *dd* signals at δ 2.46, 2.50, 2.70 and 2.74). These spectroscopic properties and its direct conversion into LNA led to structure **2** for preLNA.

The absolute configuration of the hydroxyl group at C-5 was determined as *S* on the basis of CD measurements of a derivative of preLNA. Sodium borohydride reduction of preLNA in free acid form and subsequent treatment with diazomethane yielded two epimeric diol methyl esters, **3** and **4**, in the ratio 4:1. The relative configuration of the two hydroxyl groups at C-3 and C-5 were determined as equatorial–equatorial and axial–equatorial in **3** and **4**, respectively, on the basis of their coupling constants in the ^1H NMR spectra and of the observation of NOE between 3-H and 5-H in **3**. Diol ester **4** was converted to the

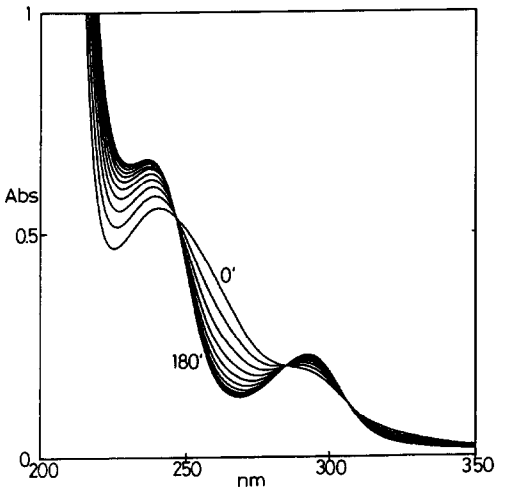

Fig. 1. Repeatedly scanned UV spectra of prelunularic acid (2) in water at pH 12. The spectra were scanned every 20 min.

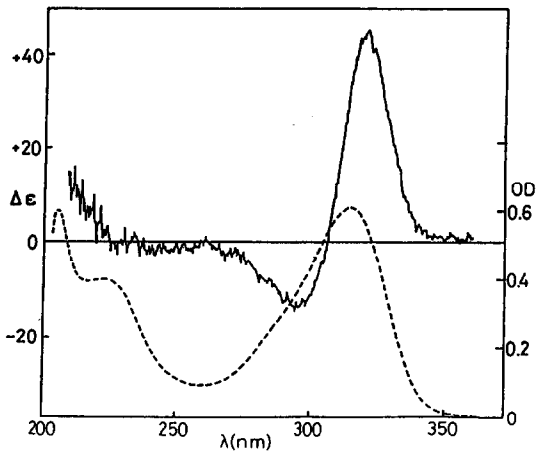

Fig. 2. CD and UV spectra of the bis(*p*-dimethylaminobenzoate) of the diol methyl ester 4 in methanol. (——) CD; (– – – –) UV spectra.

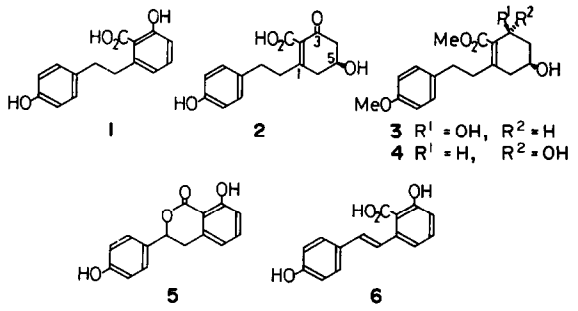

Table 1. Prelunularic acid in liverworts

Species	PreLNA (μg/g fr. wt)
Porella densifilia	25–390
Porella vernicosa	12–19
Plagiochila acanthophylla subsp. *japonica*	29
Makinoa crispata	n.d.*
Conocephalum conicum	85
Marchantia paleacea var. *diptera*	210

*Not detected.

corresponding bis (*p*-dimethylaminobenzoate) by refluxing with *p*-dimethylaminobenzoyl chloride in tetrahydrofuran in the presence of triethylamine for 24 hr. The benzoate obtained showed typical positive exciton-split CD Cotton effects (Fig. 2). The long axes of the two *p*-dimethylaminobenzoate chromophores thus constitute a right-handed screwness, and this defines the absolute configurations at C-3 and C-5 as *R* and *S*, respectively [7]. The chirality between the two benzoate transition moments are the same and independent of the ring conformation, except for an unlikely skewed boat conformation in which the two benzoate groups are axial. In fact, the bis (*p*-dimethylaminobenzoate) was proved to retain the same conformation in the cyclohexene ring as in the diol methyl ester **4** by the coupling pattern of signals at δ 6.19 (*t*-like *s*, $W_{1/2}$ = 7 Hz, 3_{eq}-H) and 5.36 (broad peak, $W_{1/2}$ = 22 Hz, 5_{ax}-H) in its ^{1}H NMR spectrum.

The analysis of preLNA in the Sep-Pak C_{18}-treated aqueous fraction of some liverworts was carried on a reversed-phase HPLC and on silica gel HPTLC by comparison with an authentic sample. As shown in Table 1, 12–390 μg/g fr. wt of preLNA was detected in all the liverworts examined, except for *Makinoa crispata* (Metzgeriales). One sample of *Porella densifolia* contained extremely high amounts of preLNA (390 μg/g fr. wt), indicating that the conditions in which the plants had been grown may affect the content of this compound. The amount of preLNA in the liverworts of Marchantiales is generally higher than that in Jungermanniales. This is consistent with the reported data on the LNA content in liverworts [3, 4]. These results clearly indicate that the formation and accumulation of preLNA are not limited to the cells of *M. polymorpha* cultured under specific conditions, but are general to liverworts.

On the basis of these facts, preLNA is a more plausible immediate precursor of LNA than stilbenoids such as hydrangenol (**5**) or its isomer hydrangeic acid (**6**). These stilbenoids isolated from *Hydrangea* species have been postulated as the immediate precursors in the biosynthesis of LNA (**1**) [8]; however, neither **5** nor **6** has ever been found in a liverwort. Furthermore, preLNA is a reasonable condensation product of *p*-coumaroyl CoA, or more preferably of dihydro *p*-coumaroyl CoA and malonyl CoA [9, 10], and is the first example of an intermediate with a 'pre-aromatic' structure which is presumed to be formed during the construction of an aromatic ring of polymalonate origin in the phenylpropanoid–polymalonate pathway [11].

EXPERIMENTAL

Plant materials. The cells of *M. polymorpha* were cultured in a modified Murashige–Skoog medium (MSK-2 medium) as

reported previously [12, 13]. Intact liverworts were collected at a small valley in Takatsuki, Osaka prefecture.

Isolation and purification of prelunularic acid (preLNA, 2). Suspension cells of *M. polymorpha* cultured for 14 days were harvested by filtration (80 g fr. wt) and extracted several times with 90% MeOH under brief heating. After removal of MeOH under red. pres., the residual aq. soln was extracted with Et_2O to remove lipophilic components. The aq. fraction obtained was proved to be free from LNA by HPLC (JASCO, Trirotor) on a Partisil 10 ODS II column (4 mm i.d. × 25 cm) eluted with MeCN–H_2O–HOAc (50:50:0.1) at a flow rate of 1 ml/min, monitoring with UV at 285 nm. This fraction was applied to a Sephadex LH-20 column and eluted with H_2O. A small portion (0.1 ml) of each chromatographic fraction was heated with 0.5 M H_2SO_4 (0.5 ml) for 1 hr and examined for LNA by HPLC. The fractions which gave LNA on acid treatment were combined and evapd to a small vol. (*ca* 2 ml). The residue was applied to a Waters Sep-Pak C_{18} cartridge and eluted successively with H_2O (2 ml), 10% (2 ml) and 60% aq. MeCN (2 ml). The fraction eluted with 10% MeCN contained preLNA since it gave LNA upon treatment with dil. H_2SO_4. The presence of LNA in the last fraction without acid treatment indicated the spontaneous formation of LNA during these purification processes. PreLNA was further purified on cellulose (Whatman, CF-11) followed by Sephadex LH-20 CC (H_2O). Approximately 2 mg of preLNA was obtained as a non-crystalline solid. Final purification was carried out by HPLC (Partisil 10 ODS II column, 8 mm i.d. × 50 cm) eluting with MeCN–H_2O (1:9) at a flow rate of 2 ml/min, monitoring with UV at 257 nm. PreLNA was eluted at 11.0 min. The R_f value of preLNA on HPTLC (Merck, Kieselgel 60F 254) was 0.6 when developed with $CHCl_3$–MeOH–H_2O (6:4:1).

PreLNA (**2**). IR ν_{max}^{film} cm^{-1}: 3300, 1653, 1610, 1600, 1573, 1504, 1402, 830. UV $\lambda_{max}^{H_2O}$ nm (log ε): at pH 7, 257 (4.02), 220 (4.00); at pH 2, 280 (sh), 240 (3.98), 222 (4.08); at pH 12 after 3 hr, 238 (4.25), 293 (3.72). EIMS *m/z* (rel. int.): 107 (100), 258 (30), which was identical to that of LNA. ^{1}H NMR (360 MHz, D_2O): δ 2.46 (1H, *dd*, *J* = 10.5, 19.5 Hz, 4_{ax}-H), 2.50 (1H, *dd*, *J* = 8.0, 21.0 Hz, 6_{ax}-H), 2.70 (1H, *dd*, *J* = 5.0, 21.0 Hz, 6_{eq}-H), 2.74 (1H, *dd*, *J* = 5.0, 19.5 Hz, 4_{eq}-H), 2.54–2.63 (2H, m), 2.75–2.83 (2H, *m*), 4.26 (1H, *br s*, 5-H), 6.86 and 7.21 (each 2H, *d*, *J* = 8.6 Hz).

Methylation of preLNA (**2**). Brief treatment of an Et_2O soln of preLNA in free acid form with CH_2N_2 yielded a methyl ester; IR ν_{max}^{film} cm^{-1}: 3300, 1735. ^{1}H NMR (100 MHz, $CDCl_3$): δ 2.4–2.9 (8H, *m*), 3.78 (3H, *s*, $-CO_2Me$), 4.22 (1H, *br* peak), 6.73 and 7.02 (each 2H, *d*, *J* = 8.6 Hz). High resolution MS *m/z* (rel. int.): $[M]^+$ 290.1133 (11.2; calc. for $C_{16}H_{18}O_5$: 290.1149), 272.1076, $[M-H_2O]^+$ (23.6; calc. for $C_{16}H_{16}O_4$: 272.1044), 258.0915 (35.7; calc. for $C_{15}H_{14}O_4$: 258.0888), 107.0503 (100; calc. for C_7H_7O: 107.0495).

Formation of diol methyl esters **3** *and* **4**. A crude mixture of preLNA in H_2O which contained *ca* 100 mg of preLNA in free acid form was added dropwise to an aq. soln of a large excess of $NaBH_4$ at room temp. After stirring overnight, the reaction mixture was acidified to pH 2 with conc. HCl and extracted with EtOAc. Evapn of the solvent afforded a mixture of diols **3** and **4** (67 mg), which was treated directly with CH_2N_2 in Et_2O overnight. The product (52 mg) was purified by flash silica gel CC eluting with *n*-hexane–EtOAc–Me_2CO (2:7:1) to give diol methyl esters **3** (18.9 mg) and **4** (5.0 mg). **3**: IR ν_{max}^{film} cm^{-1}: 3400, 1710, 1610, 1512, 1245. UV λ_{max}^{MeOH} nm (log ε): 225 (4.17), 269 (sh), 278 (3.24), 284 (3.17). ^{1}H NMR (360 MHz, MeOH-d_4): δ 1.71 (1H, *ddd*, *J* = 7.9, 10.0, 12.5 Hz, 4_{ax}-H), 2.19 (1H, *dddd*, *J* = 1.6, 3.0, 5.9, 12.5 Hz, 4_{eq}-H), 2.24 (1H, *ddd*, *J* = 2.4, 7.6, 17.6 Hz, 6_{ax}-H), 2.40 (1H, *br ddt*, *J* = 1.3, 1.6, 4.9, 17.6 Hz, 6_{eq}-H), 2.48–2.81 (4H, *m*), 3.74 and 3.80 (each 3H, *s*), 3.89 (1H, *dddd*, *J* = 3.0, 4.9, 7.6, 10.0 Hz, 5-H), 4.64 (1H, *br t*, *J* = 1.3, 2.4, 5.9, 7.9 Hz, 3-H), 6.86 and 7.12 (each 2H, *d*, *J* = 8.6 Hz). **4**: IR ν_{max}^{film} cm^{-1}: 3350, 1710, 1610, 1515, 1245. UV λ_{max}^{MeOH} nm (log ε): 224 (4.19), 269 (sh), 278 (3.28), 284 (3.21). ^{1}H NMR (360 MHz, MeOH-d_4): δ 1.62 (1H, *ddd*, *J* = 4.4, 11.2, 12.7 Hz, 4_{ax}-H), 2.05 (1H, *ddt*, *J* = 1.3, 3.4, 12.7 Hz, 4_{eq}-H), 2.07 (1H, *br dd*, *J* = 0.6, 9.1, 18.0 Hz, 6_{ax}-H), 2.56 (1H, *br dd*, *J* = 1.2, 1.3, 5.2, 18.0 Hz, 6_{eq}-H), 2.56–2.86 (4H, *m*), 3.77 and 3.80 (each 3H, *s*), 4.07 (1H, *dddd*, *J* = 3.4, 5.2, 9.1, 11.2 Hz, 5-H), 4.78 (1H, *br t*, *J* = 0.6, 1.2, 3.4, 4.4 Hz, 3-H), 6.86 and 7.15 (each 2H, *d*, *J* = 8.7 Hz).

Bis (p-*dimethylaminobenzoate*) *of* **4**. Diol methyl ester **4** (1 mg) in dry THF (5 ml) was reacted with *p*-dimethylaminobenzoyl chloride (20 mg) in the presence of Et_3N (150 mg) under reflux for 24 hr. After usual work-up, the reaction product was chromatographed on a silica gel column eluting with *n*-hexane–EtOAc (3:1), followed by further purification by HPLC (Partisil 10 ODS II, 4 mm i.d. × 25 cm) eluting with MeOH–H_2O (4:1), and monitoring with UV at 310 nm. EIMS *m/z* (rel. int.): $[M]^+$ 600, 437, 435, 270, 165, 164, 148, 121 (100). UV λ_{max}^{MeOH} nm: 223, 314. ^{1}H NMR (360 MHz, $CDCl_3$): δ 3.03 and 3.04 (each 6H, *s*), 3.63 and 3.78 (each 3H, *s*), 5.36 (1H, *br* peak, $W_{1/2}$ = 22 Hz, 5-H), 6.19 (1H, *br* peak, $W_{1/2}$ = 7 Hz, 3-H), 6.63 and 6.64 (each 2H, *d*, *J* = 9.2 Hz), 6.82 and 7.13 (each 2H, *d*, *J* = 8.6 Hz), 7.89 (4H, *d*, *J* = 9.2 Hz). CD (MeOH): $\Delta\varepsilon_{321}$ +46.3, $\Delta\varepsilon_{295}$ −14.8. In view of the minute quantity of the dibenzoate, the ε value was estimated from the UV absorbance by taking ε 31 600 as the standard value for a monobenzoate [14].

Analysis of preLNA in intact liverworts. Liverworts were extracted twice by soaking in MeOH overnight at 4°. After removal of lipophilic components, the aq. residue (*ca* 1 ml) was passed through a Waters Sep-Pak C_{18} cartridge followed by washing the cartridge with 10% aq. MeCN. The combined eluate was analysed for preLNA by HPLC (Waters μ-Bondapak C_{18}, 4 mm i.d. × 30 cm), eluting with MeCN–H_2O–HOAc (20:80:0.1) at a flow rate of 1 ml/min and monitoring with UV at 257 nm. PreLNA was eluted after 5.7 min under these conditions.

Acknowledgements—We are grateful to Dr. J. Hasegawa, Department of Agriculture, Kyoto University, for identification of plant materials and to Professor H. Kindl, Phillips University, for his interest and suggestions.

REFERENCES

1. Valio, I. F. M., Burdon, R. S. and Schwabe, W. W. (1969) *Nature (London)* **223**, 1176.
2. Fries, K., (1964) *Beitr. Biol. Pflanz.* **40**, 177.
3. Gorham, J. (1977) *Phytochemistry* **16**, 249.
4. Pryce, R. J. (1971) *Planta* **97**, 354.
5. Abe, S. and Ohta, Y. (1983) *Phytochemistry* **22**, 1917.
6. Ohta, Y., Abe, S., Komura, H. and Kobayashi, M. (1983) *J. Am. Chem. Soc.* **105**, 4480.
7. Harada, N. and Nakanishi, K. (1983) *Circular Dichroic Spectroscopy. Exciton Coupling in Organic Stereochemistry*, p. 55. University Science Books, California.
8. Pryce, R. J. (1971) *Phytochemistry* **10**, 2679.
9. Rupprich, N. and Kindl, H. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 165.
10. Fritzemeier, K.-H. and Kindl, H. (1983) *Eur. J. Biochem.* **133**, 545.
11. Weiss, U. and Edwards, J. M. (1980) *The Biosynthesis of Aromatic Compounds*, p. 326. Wiley-Interscience, New York.
12. Ohta, Y., Katoh, K. and Miyake, K. (1977) *Planta* **136**, 229.
13. Katoh, K., Ishikawa, M. Miyake, K., Ohta, Y., Hirose, Y. and Iwamura, T. (1980) *Physiol. Plant.* **49**, 241.
14. Harada, N., Suzuki, H., Uda, H. and Nakanishi, K. (1971) *J. Am. Chem. Soc.* **93**, 5577.