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Lunularic Acid and Prelunularic Acid of Liverworts in Plant Cell Culture

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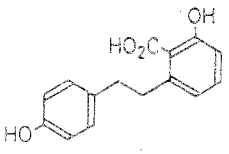
Plant hormones are low molecular weight compounds which are biosynthesized in plants in minute quantities and regulate various metabolic processes such as growth and development. Five types of plant hormones are known at present which are chemically and functionally different from each other, namely, auxins, gibberellins, cytokinins, ethylene, and abscisic acid. Among them, abscisic acid has been reported to participate in the regulation of abscission of leaves and young fruits, or dormancy of buds and seeds. The most distinct function of abscisic acid is to cause stomatal closure under water stress conditions. In addition, this compound is a potent growth inhibitor of intact plant or excised plant tissues.

Abscisic acid has been detected in all vascular plants, pteridophytes and mosses examined so far. However, this compound has never been detected in liverworts. In 1969, Valio *et al.* isolated a growth inhibiting compound from a liverwort *Lunularia cruciata* of Israel strain[1,2]. They found that this compound named lunularic acid, LNA **1**, accumulated in the liverwort in dormancy and they reported LNA as a dormancy factor in the liverwort.

Since then, LNA has been detected in more than 70 species of liverworts so far examined at concentrations of 1 - 650μg/g fresh weight[3,4]. On the basis of its growth inhibitory activity and ubiquitous occurrence in liverworts and, furthermore, from the structural similarity to



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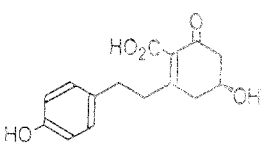

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abscisic acid, LNA was proposed as the biological equivalent of abscisic acid in higher plants[3]. Pryce suggested that the molecular size of these cpmpounds are comparable to each other and both of them carry the similar functional groups at corresponding positions.

However, we noticed that there are some obscure points about LNA to accept this compound as an endogenous growth regulator. First, the reported content of LNA, 1~650μg/g fresh weight, is extremely high. For example, 1g of fresh thallus of *Conocephalum conicum* generally contain 200~650μg of LNA[5]. If this amount of LNA was administered externally, the liverwort can never grow or will be killed in a short time. In contract, the content of ABA is in the range of 0.01~0.1μg/g fresh weight[6,7]. Second, the content of LNA has been known to fluctuate considerably depending on the analytical methods, particularly extraction procedure employed[4]. Third, none of the proposed intermediates in the biosynthesis of LNA has been detected in liverworts[8].

Thus, we decided to reexamine the behavior of LNA in liverworts. In our experiment, we

utilized the cell culture technique to obtain the plant materials. In this technique, small pieces of actively growing tissue of a plant such as yonug stem are cultivated in artificial media containing plant growth requlators like auxin and cytokinin. After continuous cell division, cell clusters called callus is formed from the original plant tissue. These undifferentiated cells grow actively and infinitely on agar medium or in liquid medium. The advantages of cell culture are simplicity, homogeneity and constant availability of experimental materials.

In the case of liverworts, gemma or sterile spores are used as the starting plant material [9~11]. The liverwort cells cultivated in nutritional medium contain high concentration of chlorophyll and grow actively under illumination. The cultured liverwort cells have been proved to have the ability to biosynthesize the similar secondary metabolites as the intact mother plant[12].

Isolation and structure determination of prelunularic acid

The effect of extraction conditions on the LNA content in liverworts was examined[13]. Extraction at high temperature or treatments under acidic or basic pH conditions increased the amount of LNA drastically. Table I shows the difference in the LNA content in cultured cells of *Marchantia polymorpha* extracted with three different procedures. In method A, the cells were extracted with boiling methanol containing 1% acetic acid. In B, the cells were extracted with boiling methanol without acid and in C, 60% acetonitrile in water was used at 4°C. These results suggest the presence of a labile compound which gives LNA under the conventional extraction procedures. This compound was isolated from the cultured cells of *M. polymorpha* according to the scheme shown in Fig. 1.

Tabla I. Prelunularic acid in liverwots

Species	PreLNA μg/g fresh wt.
Porella densifolia	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

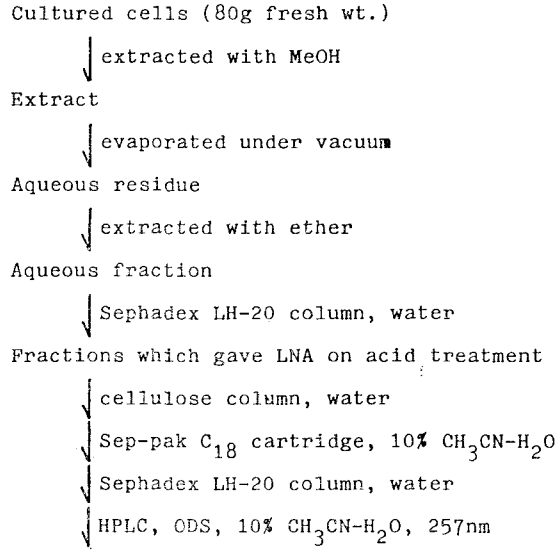

Fig. 1. Isolation of prelunularic acid.

The cells were extracted with 90% methanol and the extract was evaporated under reduced pressure. The aqueous residue was chromatographed, after removel of lipophilic compounds, on a Sephadex LH-20 column using distilled water as solvent. A small portion of each fraction was treated with diluted sulfuric acid and the fractions which gave LNA were combined. The combined fractions were further purified by successive chromatographies on reversed phase silica gel, cellulose and Sephadex LH-20 columns. Approximately 2mg of a non-crystalline compound was obtained from 80g of the fresh cultured cells. This compound was named as prelunularic

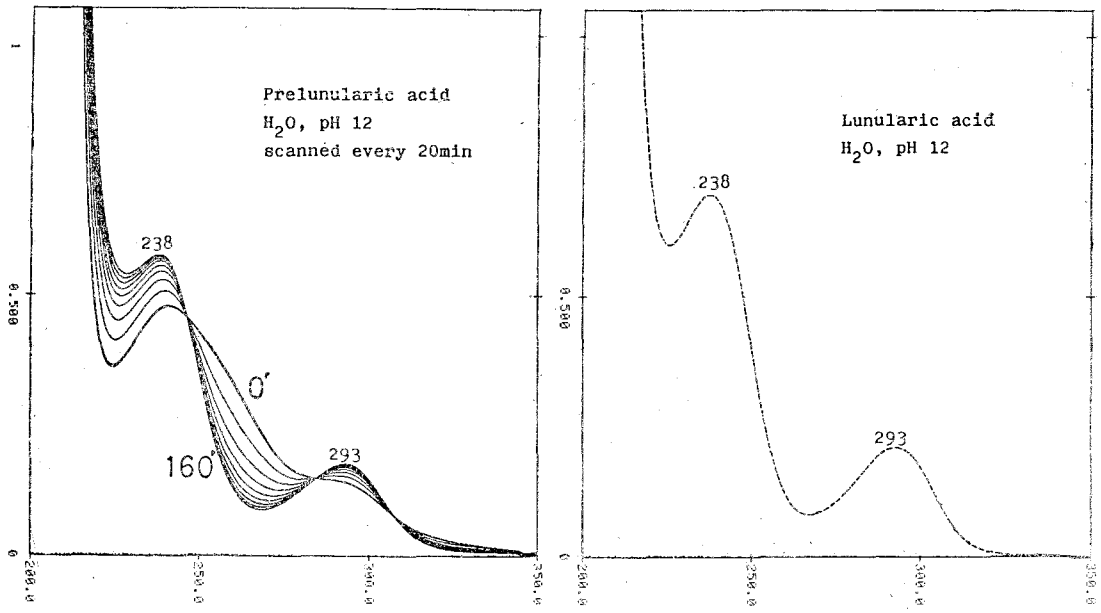

Fig. 2. Uv spectrum of preLNA and LNA.

acid, pre LNA 2.

The IR spectrum (film, cm^{-1}) of preLNA shows the presence of the following functional groups: hydroxyl (3300), aromatic ring (1600, 1514, 830), carboxylate (1573, 1402), and enone (1610). The high-resolution mass spectrum of methyl ester of preLNA showed M^+ at 290.1133 (11.2%, $C_{16}H_{18}O_5$), together with prominent peaks at 272.1076 (23.8%, $C_{16}H_{16}O_4$), 258.0195 (35.7%, $C_{15}H_{14}O_4$), and 107.0503 (100%, C_7H_7O). The ^1H NMR spectrum of preLNA (360MHz, D_2O) showed peaks at 2.4~2.8 (8H, m), 4.26 (1H, br s, CH(O−)), 6.86, and 7.21ppm (each 2H, d, J=8.6Hz, four protons on para-substituted benzene). First order analysis of the multiplet at 2.4~2.8 ppm indicated the presenc of a $CH_2CH(O-)CH_2$ moiety.

The conversion of preLNA into LNA under acidic or basic conditions was very clearly demonstrated in its UV spectrum. The UV spectrum of preLNA in water solution at pH 12 showed time-dependent changes and the final curve after about 3 hr was identical with that of LNA at the same pH conditions as shown in Fig. 2. The appearance of a clear set of isosbestic points indicated that preLNA was directly converted into LNA under this condition.

On the basis of these spectral properties and the direct conversion into LNA, the structure of preLNA was assigned as 2. The absolute configuration of the hydroxyl group at C-5 was determined in the following manner[13,14].

Reduction of preLNA with sodium borohydride and subsequent treatment with diazomethane gave two diol methyl esters. The relative configuration of the two hydroxyl groups at C-3 and C-5 in these compounds were determined by measuring of the coupling constants in ^1H-NMR spectra. The minor derivative with ax-eq hydroxyl groups was converted into corresponding bis (p-dimethylaminobenzate). The CD spectrum of this benzoate showed typical positive exciton-split CD cotton effects (Fig. 3). The long axes of the two p-dimethylaminobenzoate chromophores constitute a right-handed screwness, and this defines the absolute configurations at C-3 and C-5 as *R* and *S*, respectively.

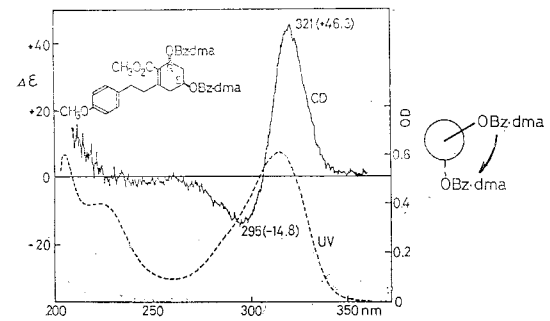

Fig. 3. Cd and uv spectrum of benzoate.

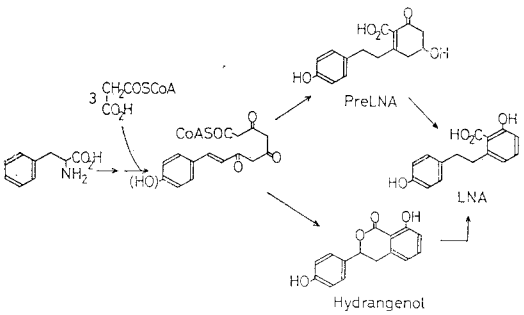

Fig. 4. Biosynthesis of lunularic acid.

The isolation of preLNA has important meaning concerning the biosynthesis of LNA itself and stilbenoids in general. First, Pryce fed the thallus of *Lunularia cruciata* with labelled phenylalanine, acetate, and hydrangenol, and concluded that LNA is biosynthesized by the phenylpropanoid-polymalonate pathway. The effective incorporation of hydrangenol, a C_{15} stilbenoid isolated from garden hydrangea, into LNA led to a conclusion that hydrangenol or its isomer, hydrangeic acid, is the immediate precursor of LNA[8]. However, in spite of the extensive efforts, hydrangenol or hydrangeic acid has never been detected in liverworts. In contrast, preLNA is a reasonable cyclization product of dihydro p-coumaryl β-triketo acid and is a plausible immediate precursor of LNA.

In the second, on the biosynthesis of stilbenoids. Stilbenoid is a large group of phenolic compounds also in higher plants. The biosynthesis of B-ring in the molecule of stilbenoid such as resveratrol has been elucidated extensively by shikimate pathway and transamination of phenylalanine as the intermediary step[16]. However, the construction of A-ring originated from three molecules of malonyl CoA is still hypothetical and any intermediate carrying pre-aromatic structure has not been isolated before. Prelunularic acid is the first example of such an intermediate with pre-aromatic structure. This indicates that the enzyme system involved in the biosynthesis of A-ring in liverwort cells is considerably different from that in higher

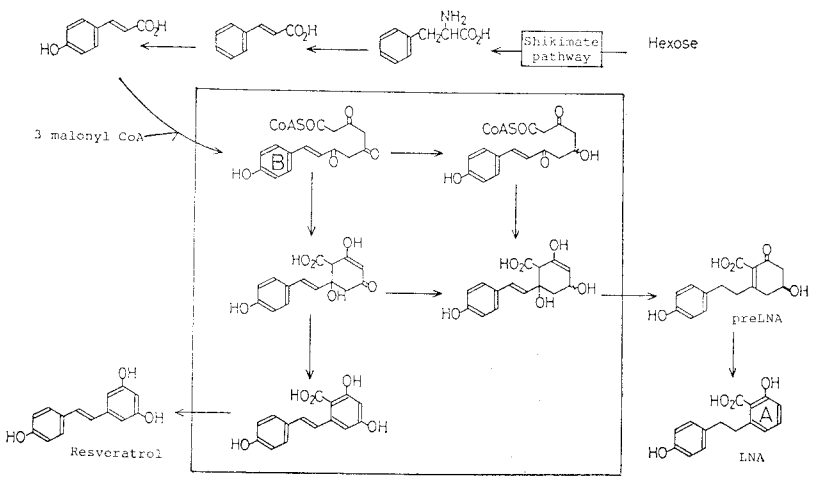

Fig. 5. Biosynthetic process of LNA and resveratrol.

plants. Stilvene synthase in higher plants seems to be a complex enzyme which catalyzes several reaction steps such as condensation of three molecules of malonyl CoA and phenylalanine-derived moiety, cyclization *via* aldol-type condensation, aromatization of the ring, and decarboxylation to give C_{14} stilbenoids such as resveratrol. On the other hand, some of reaction steps in the formation of LNA in liverwort cells are considered to proceed step by step.

Physiological behavior of preLNA and LNA

In the next, species of field grown liverworts were examined for the existence of preLNA. As shown in Table I, preLNA was detected in all liverworts examined, except for *Makinoa crispata*. This indicates that preLNA is not a metabolite which accumulates under a specific condition like cell suspension culture, but a ubiquitous component of liverworts.

As shown in Table II, the amount of LNA in the cell extracts were different depending on the extraction procedures. However, when the each extract was treated with diluted sodium hydroxide, the LNA content in thesse extracts showed similar values. This result showed that preLNA was the predominant of these two compounds. However, the LNA amount determined by extraction even at low temperature is not reliable yet, because the conversion of preLNA into LNA is still possible under this condition. Then, the real amount of LNA in liverwort materials was determined in the following manner[15].

To avoid the conversion of preLNA, this labile compound was reduced to the corresponding diols prior to the extraction with organic solvent. The frozen plant material was homogenized in ethanol in the presence of a large excess of sodium borohydride. Under this extraction condition, preLNA was reduced to the diols in fairly short time. LNA itself was stable and recovered completely after the same treatment. Table III shows the LNA content in cultured cells of *M.*

Table II. LNA content in liverworts

	extraction method	LNA content: before NaOH (a)	LNA content: after NaOH (b)	a/b×100
		μg/mg dry weight (μg/g fresh weight)		%
Marchantia polymorpha				
cultured cells	A	4.4 ±0.1	4.9 ±0.0	89.8
	B	0.39±0.01	4.9 ±0.2	8.0
	C	0.12±0.01	3.37±0.08	3.6
Conocephalum conicum				
thallus		0.046±0.001 (8.7±0.2)	1.01±0.02 (193±3)	4.6
Marchantia paleacea var. *diptera*				
thallus		0.016±0.003 (6.2±1.1)	0.81±0.02 (309±7)	1.9

Cultured cells of *M. polymorpha* were extracted by three methods, A: boiling MeOH containing 1% acetic acid, B: boiling MeOH, C: sonication in 60% acetonitrile. The thallus of *C. conicum* and *M. paleacea* were extracted by soaking in MeOH at 4°C. These extracts were assayed for LNA content before (a) and after (b) alkaline treatment (1M-NaOH at room temperature for 10min.). Each value is the mean of four samples with s.e.

Table III. LNA content determined in the presence of $NaBH_4$

	LNA content				a/b′×100
	$NaBH_4$ treatment		control		
	(a)	(b)	(a′)	(b′)	
	μg/mg dry wt				%
Marchantia polymorpha					
cultured cells	0.004	0.005	0.127	3.97	0.1
Conocephalum conicum					
thallus	0.002	0.002	0.044	1.09	0.2

Data are the means from two experiments. The cullured cells or thallus were ground with or without (control) $NaBH_4$. LNA contents in these homogenates were determined before (a and a′) and after (b and b′) alkaline treatment.

polymorpha and intact thallus of *C. conicum* determined in this way. Only 2∼4 ng of LNA was included in 1 mg of plant materials. This is about 0.1% of the total amount of preLNA and LNA.

These results led to a conclusion that "endogenous LNA" determined in the past is practically all preLNA and to questions; what is the meaning of preLNA in liverworts? or whether preLNA plays the growth regulating role instead of LNA?

At first, we tried to examine the physiological activity of preLNA in the similar way carried out on LNA by Valio, *et al.*[1] They cultivated gemma of *Lunularia cruciata* in nutritional solutions containing different concentrations of LNA, and determined the difference in their growth. However, this assay method takes approximately one month to obtain reliable results and, because of the unstability of preLNA converting into LNA even under neutral pH conditions, it is practically impossible to estimate the effect of preLNA directly by this assay method. Thus, these questions were solved by two more biological strategies. First, to know whether the accumlation of LNAs is under specific control different from that of other general metabolites, external or internal factors which control the accumulation of LNAs in the cells were examined. Second, to obtain informa-

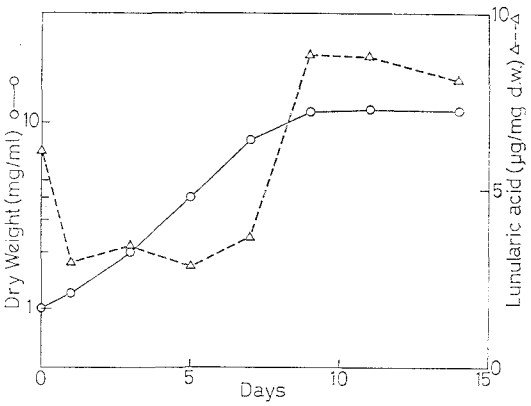

Fig. 6. Time Course of cell growth and change of LNAs content.

tions about the nature of LNAs, the intracellular distribution of LNAs was determined. LNAs is a sum of preLNA and a trace amount of LNA in plant materials.

Fig. 6 shows the time course of cell growth and in the change of LNAs content of cultured cells of *M. polymorpha*. Cell dry weight increased more than ten folds in two weeks. The content of LNAs shows a characteristic change during the culture. Immediately after transfer the cells into fresh medium, LNAs content decreased and after about 7 days, its content increased drastically. This means that the accumulation of LNAs may reflect the change in some environmental factors. For the cells cultured in liquid medium, availability of nutritional components

Table IV. Influence of removal of each major nutrients from MSK-2 medium on growth and LNA content

Removed	Dry weight		LNA content	
	mg/ml	%	μg/mg d.w.	%
None (Control)	12.9±0.1	100	6.63±0.28	100
PO_4^{3-}	8.2±0.3	64	10.6 ±0.4	160
NO_3^-, NH_4^+	4.9±0.2	38	3.58±0.13	54
Glucose	1.1±0.1	9	1.77±0.15	27

Each value is the mean of four cultures with s.e. Determination were carried out 10 days after transfer of the cells into the indicated media.

such as carbon source, nitrogen source or phosphate is one of the most influential factors. Among these major nutrients, phosphate was exhausted most rapidly and nitrogen and carbon sources were also completely exhausted from the medium by around day-10. This suggests that depletion of major nutrients may affect the accumulation of LNA and this was examined more precisely.

The cells were cultured in several different media each of which did not contain one of the major nutrients, phosphate, nitrogen or carbon source. As shown in Table IV, the deficiency of phosphate most strongly influenced the accumulation of LNAs. When the cells were cultured in the presence of different concentrations of phosphate, the LNA content started to increase immediately after phosphate was depleted (Fig. 7). It is apparent now that the content of major nutrients, particularly phosphate, affect the accumulation of LNAs[17]. The question is that this accumulation pattern is specific to LNAs or common to other general phenolic metabolites. The cultured cells of *M. polymorpha* contain, besides LNAs, phenolic compounds such as p-coumaric acid or ferulic acid, and the accumulation pattern of these phenolic compounds was proved to be identical with that of LNAs. That is, the accumulation of LNAs is not controlled under a specific regulation mechanism which is different from that for other common phenolic compounds.

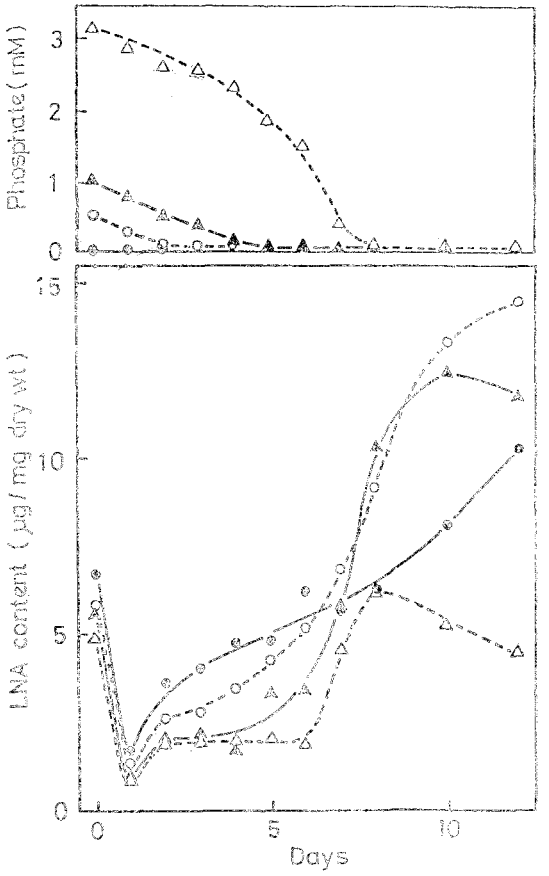

Fig. 7. The relationship between phosphate and LNA contents.

In the next, we examined the intracellular distribution of LNAs. A plant cell is composed of cotoplasm and several kinds of small organelles such as chloroplasts, mitochondria or vacuoles. Among these organelles, vacuoles have been known to contain various reserve and waste

products including phenolic compounds like tannins[18], coumarin[19] or shikimic acid[20]. Thus, it is essential to reveal whether LNAs are also distributed in vacuoles or not.

Treatment of the cultured cells of *M. polymorpha* with cell wall digesting enzyme gave protoplasts in a fairly high yield. Rupture of the protoplasts by gentle pipetting followed by application of the homogenate to discontinuous density gradient centrifugations resulted in a separation of cytoplasm, chloroplasts or vacuoles. The purities of these organella fractions were examined by the measurement of corresponding marker enzymes. Determination of LNAs amount in these organella fractions gave a result which showed that approximately 50% of LNAs was located in vacuoles and the rest was found in cytoplasm. No LNAs was detected in chloroplasts or mitochondria. This distribution pattern is totally different from that of abscisic acid and is similar to that of other common phenolic compounds. Abscisic acid has been reported to accumulate mainly in chlorpplasts[21].

Conclusion

In conclusion, liverwort cells contain a large amount of preLNA instead of LNA itself and the accumulation of this compound is enhanced under conditions which is not favorable for the growth of the cells. PreLNA and LNA are not likely plant growth regulator or plant hormone such as abscisic acid, but they are classified as one of common phenolic metabolites peculiar to liverworts.

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