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Induced Furoeudesmanes: A Defense Mechanism Against Stress in *Laggera pterodonta*, a Chinese Herbal Plant

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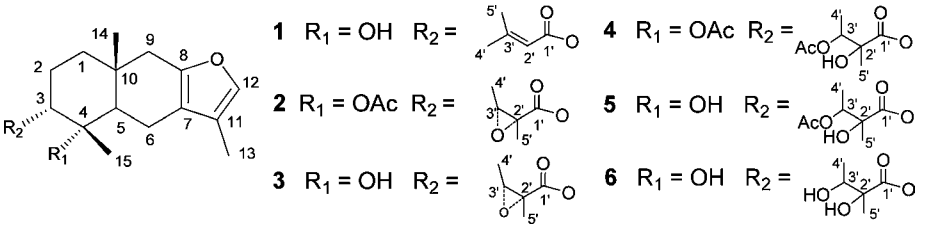
ABSTRACT



Laggera pterodonta **displays different phenotypes in its natural habitat but expresses a uniform phenotype with large, broad leaves and fewer secondary metabolites when grown under optimal conditions. The production of six furoeudesmanes is only induced when *L. pterodonta* encounters stresses, conferring host resistance against a broad spectrum of plant invaders.**

Plant secondary metabolites are compounds present in specialized cells that are not essential for cellular metabolism yet are required for the survival of the plant within its external environment.[1] These compounds are believed to assist plant defense against insect herbivory and pathogenic attack.[2] Plants have played a dominant role in traditional medicine systems, and the importance of secondary small molecular compounds in plants is significant to pharmacognosy and the development of synthetic derivatives for the pharmaceutical industry. However, these compounds did not undergo their evolutionary selection based on human therapeutic agents, rather environmental defensive systems, but what causes plants to synthesize many different secondary metabolites remains largely unexplored. Therefore, the research into the causes of the induction of these downstream compounds, their structure, and the role they perform in plants is attracting a lot of attention from chemists and physiologists.

Laggera pterodonta is widely distributed in Southwest China and has been used historically in the "Yi" ethnopharmacy as an anti-inflammatory and antibacterial agent.[3] Previous investigations reported the isolation of flavonoids and eudesmanes from this plant.[4] In our pharmacological evaluation test, the main sesquiterpenoid, pterodontic acid (PA),[4d] was shown to have an anti-inflammmatory effect as observed by decreasing xylene-induced ear edema in mice by up to 64.1% at a dosage of 10 mg/kg, roughly comparable to that of aspirin at 200 mg/kg (73.7%) (Supporting Information, Table S1).

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Figure 1. Different phenotypes of *L. pterodonta*. (a) *L. pterodonta* grown in dry soil had multiple branches and narrow leaves. (b) *L. pterodonta* produced fewer branches and large broad leaves when grown in moist conditions. (c) Same phenotype of *L. pterodonta* was cultivated in a greenhouse from the seeds of the different phenotypes.

During the medicine quality control procedure, we did not find any PA in some preparations, which led to the discovery that two phenotypes of *L. pterodonta* (Figure 1a,b) exist in the wild. Both phenotypes of this plant have been used as the raw material for herbal medicines, but the plants with broad leaves did not produce PA. This finding led us to question the basis for the phenotype. Next, we collected seeds of *L. pterodonta* with different phenotypes and cultivated them in a greenhouse under optimal conditions, such as abundant moisture (50−60% RH) and appropriate temperature (25 °C). Intriguingly, all the plants grew with large broad leaves, produced fewer secondary metabolites, and became sensitive to insect attack (Figure 1c). Surprisingly, after 3 days of continual attack by insect larvae (*Neurois renalba* or *Argyrogramma agnate*), the six compounds were induced in the leaves of plants. However, after 10 days, these induced compounds gradually decreased in comcentration, possibly via degradation.

After grinding leaf tissue with their mandibles, insects (larvae) use oral secretions to transport food into their mouthparts. These oral secretions, composed of regurgitant labial and mandibular saliva, contain a mixture of potential elicitors that are recognized by the plant, which produce a defense response.[5] To determine whether the biosynthesis of the six compounds were triggered by the oral secretion of insect herbivory or simply caused by wounding,[6] we prepared *L. pterodonta* in tissue culture seedlings in an incubator and cultivated the plants in a greenhouse. Plants were divided randomly and maintained separately. Chafing and cutting were used to cause wounding (Figure 2a). Our results showed that the six compounds were induced in the leaves of all the wounded plants, and their concentrations were observed to be proportional to the degree of wounding (Figure 2b and Supporting Information).

Previous studies showed that the plants defended themselves against insect herbivory attack using chemical and physiological defenses by synthesizing and rapidly releasing volatile organic compounds (VOCs). Subsequently, adjacent plants detected the VOCs released by the

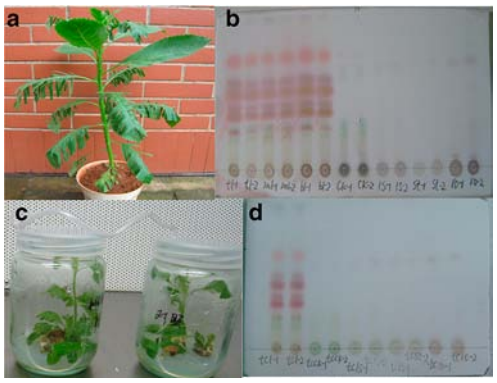

Figure 2. Inducible compounds in *L. pterodonta*. (a) Cut plant. (b) Thin layer chromatogram (TLC) profile showed that all leaves of the treated plant produced six induced compounds (red spots, lanes 1−6), whereas its leafstalk (lanes 9 and 10), stem (lanes 11 and 12), root (lanes 13 and 14), and leaves of the unwounded plant (lanes 7 and 8) did not. (c) Two culture flasks linked by polyvinyl chloride pipe; the seedling of *L. pterodonta* on the left flask was cut, while the plant was kept intact in the right flask. (d) TLC profile of *L. pterodonta* in both flasks showed the presence of induced compounds in leaves (red spots, lanes 1 and 2), but not in leafstalk (lanes 5 and 6), stem (lanes 7 and 8), root (lanes 9 and 10), and control (lanes 3 and 4).

neighboring wounded plants and then activated their own defenses before being attacked.[7] To demonstrate this association, we developed apparatus that permitted the transmission of volatile compounds from one plant to another (Figure 2c). Seedlings of *L. pterodonta* were cut in one flask, while the intact plants were growing in the connected flask. Intriguingly, we found that the six compounds were detectable in the undamaged plants, in approximately equivalent proportions to those of the wounded plants (Figure 2d).

It is well-established that jasmonate (JA) plays a prominent role in promoting plant defense responses to herbivores and wounding, while salicylic acid (SA) crucial for the plant's defense is generally involved in the establishment of systemic acquired resistance. Unlike protease inhibitor (PI) induced by JA and pathogenesis-related (PR) proteins induced by SA, plant secondary metabolite defense was related to the downstream products of stress signals.[8] However, are these induced compounds specific to the JA pathway or related to stress phytohormones? In order to test this hypothesis, we treated both cultivated plants in a greenhouse and tissue culture seedlings of *L. pterodonta*, with signal molecules of wounding (JA), pathogens (SA), and induced drought (abscisic acid and ethrel), respectively, to mimic environmental stresses (Supporting Information). Surprisingly, the six induced compounds were detected in the leaves of all the treated plants but not in the controls. Furthermore, high temperature (> 40 °C), low temperature (< 5 °C), and intense

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ultraviolet (1200 lux, 5 h) conditions could also induce the biosynthesis of these compounds in tissue culture seedlings. Evidently, the biosynthesis of these compounds was in response to a variety of stimuli.

The six induced compounds (red spots on TLC) were isolated by rapid column chromatography repeatedly at low temperature (to avoid decomposition), and they were elucidated to be furoeudesmanes by detailed analysis of their spectral data and X-ray; of which **1**, **2**, and **4**–**6** were novel (Figures S1 and S4–S38, Tables S2 and S3).

Compound **3** was determined to be 3α-(2′,3′-epoxy-2′-methylbutyryloxy)furoepaltol by comparison of its ^{1}H and ^{13}C NMR data with the literature.[9] Single-crystal X-ray diffraction further supported the structure and clarified the relative configuration of **3** (Figure S1 and Supporting Information). The ^{1}H and ^{13}C NMR spectra of **1**[10] were similar to those of **3**, except for the substituent group at C-3. Two methyl protons appeared as singlets at δ_H 1.86 and 2.14, together with an olefinic proton at δ_H 5.76 (s), and corresponding carbon signals at δ_C 117.3 (d) and 156.7 (s) in **1** suggested a 2′(3′)-ene-3′-methylbutyryloxy as the substituent,[9] which was in accord with its molecular formula of $C_{20}H_{28}O_4$. The HMBC correlation of δ_H 4.80 (m, H-3) with δ_C 166.3 (C-1′, s) further supported the substituent at C-3.

Comparison of ^{1}H and ^{13}C NMR data of **2**[11] with those of **3** suggested one more acetyl group in **2**. The HMBC correlation of δ_H 1.95 (3H, s) with a quaternary carbon at δ_C 83.9 (C-4) suggested the acetylation of 4-OH.

The ^{1}H and ^{13}C NMR spectra of **4**[12] were very similar to those of **2**, except for a pair of downfield signals at δ_C 75.8 (s), 74.4 (d) in **4** instead of δ_C 60.3 (s), 59.8 (d) in **2**, which suggested the cleavage of an epoxyl group at the substituent. An additional acetyl group (δ_C 20.1, q and 169.9, s) was connected to 3′-OH, revealed by the downfield signal at δ_H 5.03 (q, J = 6.3 Hz, H-3′), which was further supported by the HMBC correlation of δ_H 1.98 (3H, s) with δ_C 74.4 (d, C-3′).

The ^{1}H and ^{13}C NMR spectra of **5**[13] displayed similarity to those of **4** except for the presence of only one acetyl group in **5**, in accordance with its molecular formula of $C_{22}H_{32}O_7$. The acetyl group was connected to 3′-OH, supported by the HMBC correlation of downfield proton H-3′ (δ_H 5.10, q, J = 6.3 Hz) with δ_C 170.2 (s).

Table 1. Antifeeding Activity of the Induced Furoeudesmanes Against the Larvae of Four Species of Insects[a]

compounds	*A. agnate*	*S. exigua*	*P. rapae*	*P. xylostella*
1	83.5 ± 3.7	59.1 ± 4.8	42.6 ± 3.6	39.5 ± 3.2
2	92.4 ± 4.6	82.8 ± 2.4	63.4 ± 3.2	31.8 ± 2.3
3	90.5 ± 3.9	80.9 ± 4.0	44.9 ± 1.5	39.4 ± 4.6
4	96.5 ± 3.1	48.7 ± 3.6	52.2 ± 3.3	28.6 ± 3.7
5	86.8 ± 5.8	90.3 ± 1.7	72.6 ± 2.5	27.4 ± 3.5
6	76.7 ± 1.6	51.1 ± 3.7	59.9 ± 4.0	35.4 ± 2.4
azadirachtin	94.6 ± 3.8	89.7 ± 2.4	76.8 ± 3.1	46.6 ± 1.9

[a] The tested concentrations were 20 μg/leaf-disk, equal to 0.80 μg/mg fresh cabbage leaves. All values are expressed as mean ± SE for all groups (n = 3).

Table 2. Inhibitory Constants of the Induced Furoeudesmanes to Hydrolysis of Synthetic Chromogenic Substrates[a]

	inhibitory constant (K_i)	
compounds	serine protease of *Pieris rapae*	serine protease of *Drosophila melanogaster*
1	4.28×10^{-5}	3.76×10^{-5}
2	2.99×10^{-5}	3.27×10^{-5}
3	6.17×10^{-5}	4.38×10^{-5}
4	2.00×10^{-4}	8.67×10^{-5}
5	8.75×10^{-4}	7.48×10^{-5}
6	2.69×10^{-5}	5.50×10^{-5}
azadirachtin	2.88×10^{-5}	3.25×10^{-5}

[a] The concentrations of compounds were 100 μg/mL. All results are expressed as mean ± SE for all groups (n = 3).

Compound **6**[14] was shown to be a deacetylated derivative of **5** by detailed comparison of ^{1}H and ^{13}C NMR spectra of two compounds, in accordance with its molecular formula of $C_{20}H_{30}O_6$.

Observation under greenhouse conditions and the disk test revealed that the larvae of *N. renalba* preferred leaves of *L. pterodonta* without the induced compounds (Figure S3). An antifeeding evaluation of the induced furoeudesmanes was also performed against the second-instar larvae of *Argyrogramma agnate*, *Spodoptera exigua*, *Pieris rapae*, and the third-instar larvae of *Plutella xylostella*, by using the conventional leaf-disk method (Supporting Information). All six compounds showed significant antifeeding activities, in which compounds **2**, **3**, and **5** displayed an approximately equal effect to the well-known antifeedant, azadirachtin (Table 1). Specifically, the induced compounds inhibit the digestion of plant proteins by the insects, as suggested by the result of the serine protease (from the midgut of the larvae) inhibition assay (Table 2).

During our 5 year observation, we have not seen any *L. pterodonta* plants that have been infected by pathogens

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(10) Compound **1**: white oil; TLC (petroleum ether/Me_2CO, 3:1 v/v); R_f = 0.55; IR (film) ν_{max} 3499, 2925, 1716, 1233, 1082 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables S2 and S3, respectively; HRESIMS (m/z) $[M + H]^+$ calcd for $C_{20}H_{29}O_4$ 333.2065, found 333.2074.

(11) Compound **2**: colorless oil; TLC (petroleum ether/Me_2CO, 3:1 v/v); R_f = 0.47; IR (film) ν_{max} 2933, 1752, 1247, 1143 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables S2 and S3, respectively; HRESIMS (m/z) $[M + Na]^+$ calcd for $C_{22}H_{30}O_6Na$ 413.1940, found 413.1927.

(12) Compound **4**: white amorphous powder; TLC (petroleum ether/Me_2CO, 3:1 v/v); R_f = 0.38; IR (KBr) ν_{max} 3441, 2942, 1739, 1245, 1065 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables S2 and S3, respectively; HRESIMS (m/z) $[M + H]^+$ calcd for $C_{24}H_{35}O_8$ 451.2331, found 451.2340.

(13) Compound **5**: white amorphous powder; TLC (petroleum ether/Me_2CO, 3:1 v/v); R_f = 0.33; IR (KBr) ν_{max} 3503, 2938, 1742, 1254, 1067 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables S2 and S3, respectively; HRESIMS (m/z) $[M + H]^+$ calcd for $C_{22}H_{33}O_7$ 409.2226, found 409.2213.

(14) Compound **6**: white amorphous powder; TLC (petroleum ether/Me_2CO, 3:1 v/v); R_f = 0.25; IR (KBr) ν_{max} 3441, 2938, 1773, 1247, 1270 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables S2 and S3, respectively; HRESIMS (m/z) $[M + H]^+$ calcd for $C_{20}H_{31}O_6$ 367.2120, found 367.2122.

Table 3. TMV Infection Inhibition Activities of the Induced Furoeudesmanes on *N. glutinosa*[a]

compounds	inhibition rate (%)	compounds	inhibition rate (%)
1	52.9 ± 2.1	**5**	43.1 ± 2.2
2	50.8 ± 2.6	**6**	58.1 ± 3.4
3	44.3 ± 1.6	ningnanmycin	58.6 ± 2.0
4	39.9 ± 2.1		

[a] The concentrations of compounds ranged from 0.01 to 10 μM.

in the wild or in our greenhouse. Furthermore, plants were infected by artificial inoculation with common agricultural pathogens. Tobacco mosaic virus (TMV), one of the most well-studied plant viruses, infecting more than 400 plant species that belong to 36 families,[15] was selected to evaluate the antiviral activity of the six induced furoeudesmanes from *L. pterodonta*. These induced compounds exhibited a marked inhibition activity against TMV replication at the local lesion host in *Nicotiana glutinosa* by the half-leaf method, comparable to ningnanmycin (Table 3 and Supporting Information).

The leaf-disk method was also used to evaluate the inhibition of the induced furoeudesmanes on TMV replication in the systemic infection host, *N. tabacum* cv.K326. The concentration of TMV was assayed by triple antibody sandwich ELISA (TAS-ELISA), and the accumulation of TMV coat protein (CP) was tested by Western blot analysis (Figure 3 and Supporting Information). The result showed that all the compounds inhibited TMV replication, with compounds **1**, **2**, and **6** reducing the accumulation of the TMV CP significantly (Figure 3).

The concentrations of six compounds are proportional to the degree of wounding and decomposition. To further quantify the concentrations of compounds, samples were analyzed by HPLC, and we found that their contents could reach a maxima of 0.23% (**1**), 0.20% (**2**), 0.43% (**3**), 0.081% (**4**), 0.27% (**5**), and 0.16% (**6**) in fresh leaf, respectively (Figure S2). These concentrations were apparently higher than those needed to prevent the antifeeding and antivirus effects (Tables 1–3).

Our observation that *L. pterodonta* with large broad leaves produces fewer secondary metabolites when grown in optimal conditions provides a suitable model to understand the important balance between the allocation of energy for growth and secondary metabolites required for the synthesis of defense molecules. The biosynthesis of the six induced furoeudesmanes in *L. pterodonta* was not triggered until the plant encountered one of many stimuli.

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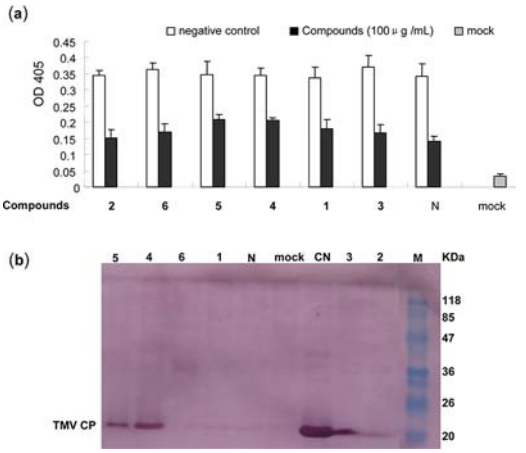

Figure 3. Inhibitory effects of compounds **1**–**6** (100 μg/mL) against TMV replication in *N. tabacum* cv.K326. (a) Replication of TMV was determined by TAS-ELISA. All results are expressed as mean ± SE (n = 3). (b) TMV coat protein (CP) was measured by Western blot. Lane M refers to protein marker; lane N refers to treatment with ningnanmycin (100 μg/mL); lane CN means control; other lanes refer to treatment with the six compounds, respectively.

However, these secondary metabolites have a role to assist the plant to confer host resistance against insects and viruses. In this way, the induction of secondary metabolites could perform a nonspecific and secondary defensive role in augmenting other well-known responses, such as the systemic wound response and systemic acquired resistance. Additionally, our finding suggests that favorable conditions for plant growth may not be good for the accumulation of the secondary metabolites in the production of medicinal plants.

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Supporting Information Available. Supplementary text, Figures S1–S38, Tables S1–S3, refs S1–S12, and X-ray crystallographic data (CIF file) of compound **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.