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INDOLOPYRIDOQUINAZOLINE, FUROQUINOLINE AND CANTHINONE TYPE ALKALOIDS FROM *PHELLODENDRON AMURENSE* CALLUS TISSUES

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Key Word Index—*Phellodendron amurense*; Rutaceae; callus tissue; canthin-6-one; indolopyridoquinazoline alkaloids; furoquinoline alkaloids; anthranilic acid; chemotaxonomy.

Abstract—Callus tissue from the stems of *Phellodendron amurense* (Rutaceae) produced the indolopyridoquinazoline type alkaloids, rutaecarpine, 7,8-dehydrorutaecarpine, which is new from a natural source, canthin-6-one, and three furoquinoline alkaloids, dictamnine, γ-fagarine and skimmianine, as the main alkaloid components, along with a small amount of three isoquinoline alkaloids (berberine, palmatine and magnoflorine).

The indolopyridoquinazoline and furoquinoline type alkaloids are isolated for the first time from *Phellodendron amurense*.

INTRODUCTION

The bark of *Phellodendron amurense* Rup., (amur cork tree), which belongs to the family Rutaceae, is used as a crude drug in Japan and China as an anti-stomachic, for intestinal function control, and as an anti-inflammatory and anti-pyretic agent. The major chemical constituents in the bark are the isoquinoline alkaloids berberine, palmatine, jatrorrhizine and magnoflorine [1]; flavone glucosides have also been isolated from the leaves [2], together with phytosterols [3] and limonoidal triterpenes (obakunone and γ-hydroxybutenolides) and more recently, twelve phenolic compounds, were reported from an aqueous extract of *Phellodendron* bark [4].

The report describes the establishment of callus tissue from the stem of *P. amurense* and the structural elucidation of indolopyridoquinazoline, canthinone and furoquinoline type alkaloids present in callus tissues.

RESULTS AND DISCUSSION

Calli were established in April 1992 from the stem of *P. amurense* Rup. (Rutaceae) on MS medium and were subcultured at 5-week intervals in the dark on MS medium containing 2,4-D (1 mg/l) with kinetin (KIN, 0.1 mg/l) as plant growth regulators. The stored calli (fr. wt 4.2 kg, dry wt 130 g), subcultured over 7–8 months at 5-week intervals, were extracted successively with MeOH and EtOAc and both extracts combined. The extracts were next chromatographed on a silica gel column to give three indole alkaloids (5–7), three furoquinoline alkaloids (8–10), and small amounts of the known isoquinoline alkaloids, berberine (**1**), palmatine (**2**), and magnoflorine (**4**).

Alkaloid **5** was obtained as a pale yellow powder. Its structure was determined by a comparison of its UV, IR, MS, ^{1}H and ^{13}C NMR spectral data with those in the literature [5] and by direct comparison with authentic canthin-6-one (^{1}H NMR and MS) [6]. The ms spectrum of alkaloid **6** exhibited a $[M]^+$ at *m/z* 287 and its ^{1}H NMR spectrum exhibited two methylene signals at δ 3.24 (2H, *t*, *J* = 7 Hz, H-8) and 4.6 (2H, *t*, *J* = 7 Hz, H-7) ppm, eight aromatic proton signals at δ 7.20–8.35 (8H), and 9.15 (1H, *bs*, NH) ppm. Further, eighteen carbon signals corresponding to the indolopyridoquinazoline skeleton were observed in the ^{13}C NMR spectrum (Table 1). The structure of alkaloid **6** was determined to be rutaecarpine [7] by direct comparison with an authentic sample [7], and this is the first report from this plant source.

Alkaloid **7** exhibited a molecular ion $[M]^+$ at *m/z* 285 which was 2 mass units less than that of rutaecarpine **6**. Its UV spectrum showed the presence of a highly conjugated system (λ_{max} nm (EtOH) 217, 250, 281, 330, 348, 368 and 390), and its IR (KBr)

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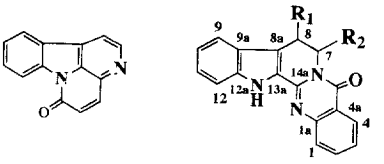
Table 1. ^{1}H NMR and ^{13}C NMR assignments and ^{13}C-^{1}H long-range correlations of **6** and **7** by HMQC, HMBC and HOHAHA experiments

	(6)			(7)		
	δ_C	δ_H	Cross peaks in HMBC spectrum	δ_C	δ_H	Cross peaks in HMBC spectrum
C-1	126.7	(7.66)	126.2 (C-3), 121.2 (C-4a)	126.0	(7.81)	116.7 (C-4a)
C-1a	147.6			147.5		
C-2	134.3	(7.72)	147.5 (C-1a), 127.3 (C-4)	134.9	(7.81)	147.5 (C-1a), 127.7 (C-4)
C-3	126.2	(7.43)	126.7 (C-1), 121.2 (C-4a)	124.8	(7.46)	126.0 (C-1)
C-4	127.3	(8.32)	161.6 (C-5), 147.5 (C-1a), 134.3 (C-2)	127.7	(8.50)	159.2 (C-5), 147.5 (C-1a), 134.9 (C-2)
C-4a	121.2			116.7		
C-5	161.6			159.2		
C-7	41.1	(4.59)	161.6 (C-5), 144.9 (C-14a), 118.3 (C-8a)	118.8	(8.75)	159.2 (C-5), 140.1 (C-14a), 120.9 (C-8a), 107.7 (C-8)
C-8	19.7	(3.52)	144.9 (C-14a), 127.2 (C-13a), 125.7 (C-9a)	107.7	(7.55)	122.5 (C-9a), 118.7 (C-7)
C-8a	118.3			120.9		
C-9a	125.7			122.5		
C-9	120.1	(7.65)	138.2 (C-12a), 125.6 (C-11)	120.9	(8.02)	139.5 (C-12a), 127.4 (C-11), 120.9 (C-8a)
C-10	120.7	(7.18)	125.7 (C-9a), 112.1 (C-12)	121.3	(7.33)	122.5 (C-9a), 112.3 (C-12)
C-11	125.6	(7.33)	138.2 (C-12a), 120.1 (C-9)	127.4	(7.48)	139.5 (C-12a), 120.9 (C-9)
C-12	112.1	(7.42)	125.7 (C-9a), 120.7 (C-10)	112.3	(7.52)	122.5 (C-9a), 121.3 (C-10)
C-12a	138.2			139.5		
C-13a	127.2			134.6		
C-14a	144.9			140.1		

In $CDCl_3$.

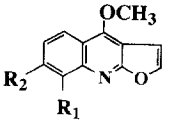


(5)

R_1 R_2

(6) H H

(7) Δ



(8) $R_1 = H$ $R_2 = H$

(9) $R_1 = OCH_3$ $R_2 = H$

(10) $R_1 = OCH_3$ $R_2 = OCH_3$

Fig. 1. Alkaloids from *Phellodendron amurense* callus tissue.

spectrum exhibited absorption bands at 1690 and 1600 cm^{-1}, indicative of a carbonyl group. Further, instead of signals for two methylene groups present in rutaecarpine, two new aromatic proton resonances at δ 7.55 (1H, *d*, $J = 8$ Hz, H_8) and 8.75 (1H, *d*, $J = 8$ Hz, H_7) ppm, were observed in the ^1H NMR spectrum.

Full assignments of the protons and protonated carbons of alkaloid 7 were attained by ^1H-^1H COSY, HOHAHA and ^1H-^{13}C heteronuclear correlated 2D NMR spectra (HMQC). Furthermore, a NOE effect was also observed between NH (δ 10.15) and H_{12} (δ 7.52), and the HMBC spectrum showed a significant cross-peak, due to a $^3J_{C\text{-}H}$ correlation, between H_4 (δ 8.50) and C-5 (δ 159.2, C=O). Accordingly, the following connectivities C_1—C_2—C_3—C_4 and C_9—C_{10}—C_{11}—C_{12} were established starting from the well resolved signals at H_4 (δ 8.50) and H_{12} (δ 7.52), respectively by a combination of COSY, HOHAHA and HMQC experiments. Furthermore, by a proton detected multiple bond ^1H-^{13}C correlation spectrum (HMBC) all of the quaternary carbon signals were assigned (Table 1). From these data, the structure of **7** was suggested to be 7,8-dehydrorutaecarpine, and this was established by comparing the spectral data (^1H NMR, UV and IR) with those published [8]. This compound has been reported as a synthetic product [8] from rutaecarpine, but this is its first isolation from a natural source.

Three known furoquinoline-type alkaloids, dictamnine (**8**) [9], γ-fagarine (**9**) [10, 11] and skimmianine (**10**) [12] were also isolated and identified by ^1H NMR, ^{13}C NMR, and mass spectrometry, and by comparison of spectral data with those published [13, 14]. The alkaloids produced from the callus tissue are shown in Fig. 1. The canthinone and indolopyridoquinazoline type alkaloids (5–7) and the three furoquinoline type alkaloids (8–10), along with a small amount of the known protoberberine type alkaloids, berberine (1), palmatine (2), and the aporphine type, magnoflorine (4) have been produced from the callus tissue. However, jatrorrhizine (3), obtained from the original plant, was not detected, this being somewhat surprising given its occurrence in ranunculaceous, berberidaceous and menispermaceous callus tissue [15].

Callus tissues (fr. wt 8.5 kg, dry wt 220 g) subcultured after 4 y from establishment of the callus, were next re-checked for the reproducibility of alkaloid production. The same alkaloids (**1**, **2**, **4**–**10**) obtained from the previous cultured callus tissue (after 2 y from establishment of the callus) were again produced. That is the biosynthetic ability for production of the alkaloids by the callus was retained for 5 y following callus establishment. Since the alkaloids **5**, **6**, **7**, **8**, **9** and **10** have not been reported previously as constituents of the original plant, *P. amurense*, it was next of interest to compare the biosynthetic capability between the callus and the plant, and to reinvestigate whether the canthinone, indolopyridoquinazoline and furoquinoline type alkaloids were present in the original plant.

In this context, a previous investigation of the constituents of the fine roots and aerial parts of a young intact plant (*P. amurense*, 1-year-old from germination) collected from cultivated field in October 1994, *not generated from the callus*, afforded a small amount of canthin-6-one (**5**) (0.04%, dry wt), and larger amounts of berberine (**1**), palmatine (**2**), jatrorrhizine (**3**) and magnoflorine (**4**) [16]. However, the indolopyridoquinazoline type alkaloids, rutaecarpine (**6**) and 7,8-dehydrorutaecarpine (**7**), and the furoquinoline type alkaloids, dictamnine (**8**), γ-fagarine (**9**), and skimmianine (**10**), which were produced as major alkaloid products in the callus tissues, were not detected. Additionally, the ripe fruits of *P. amurense* were collected in October 1996 and extracted with MeOH in order to compare their alkaloid compositions with the callus. As a result of this investigation, the isoquinoline alkaloids, berberine (150 mg 0.036% dry wt), palmatine (trace amount) and magnoflorine, and also rutaecarpine (25 mg, 0.0061% dry wt), were identified by TLC comparison with the standard samples and also from their spectral data (^1H, ^{13}C NMR and MS). However, as before, furoquinoline (skimmianine, dictamnine and γ-fagarine) and canthin-6-one alkaloids were not detected.

In summary, a comparison of the alkaloids occurring in the callus tissues and the plant is shown in Table 2. As shown, the callus tissues produced indolopyridoquinazoline, canthinone and furoquinoline type alkaloids as its main alkaloids. It can, therefore, be presumed that in the callus tissue the biosynthetic pathway to the indole alkaloids from chorismic acid via anthranilic acid is activated more than that to the

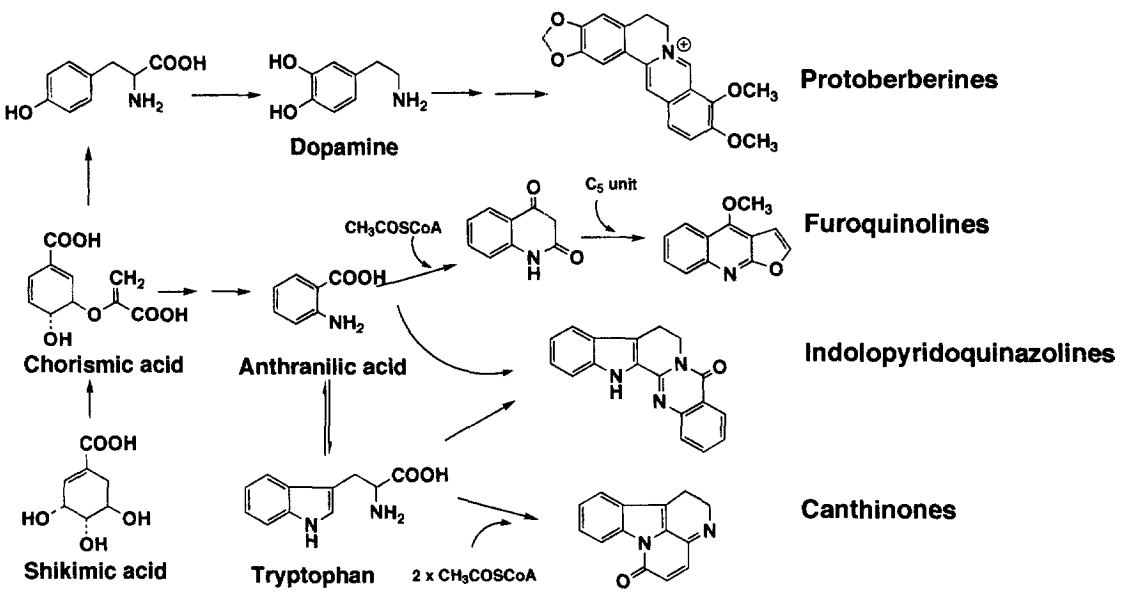
Table 2. Comparison of alkaloids in callus tissue and plant of *Phellodendron amurense*

Type of Alkaloids	Callus	Plant			
		Stem	Root	Fruit	Bark*
Protoberberine type					
Berberine	+	+	+	+	+
Palmatine	+	+	+	+	+
Jatrorrhizine	−	+	+	−	+
Aporphine type					
Magnoflorine	+	+	+	+	+
Furoquinoline type					
Dictamnine	+	−	−	−	−
γ-Fagarine	+	−	−	−	−
Skimmianine	+	−	−	−	−
Canthinone type					
Canthin-6-one	+	+	+	−	−
Indolopyridoquinazoline type					
Rutaecarpine	+	−	−	+	−
7,8-Dehydrorutaecarpine	+	−	−	−	−

(−) absent (+) present * commercial crude drug.

isoquinoline type alkaloids from chorismic acid via the prephenic acid pathway in the plant. The individual differences of the metabolic ability to form the indole or isoquinoline type alkaloids from chorismic acid were, therefore, probably affected by the growth conditions of the dedifferentiated callus tissues and its plant, respectively (Scheme 1), although the reasons are as yet unknown.

The canthin-6-one alkaloid **5** was previously found exclusively in the families Rutaceae and Simaroubaceae of the Rutales [17]. However, the distribution of indolopyridoquinazoline alkaloids, having the more complex structure, such as rutaecarpine (**6**), appears to be restricted to the genera *Euodia* and *Zanthoxylum* of the Rutaceae [18]. It is interesting from a chemotaxonomic point of view that the indolopyridoquinazoline type alkaloids are produced as the main alkaloids from *Phellodendron amurense* callus tissue. Further work is in progress in an attempt to enhance their production.



Scheme. 1. Biosynthetic pathway of chorismic acid metabolism in callus tissue of *Phellodendron amurense*.

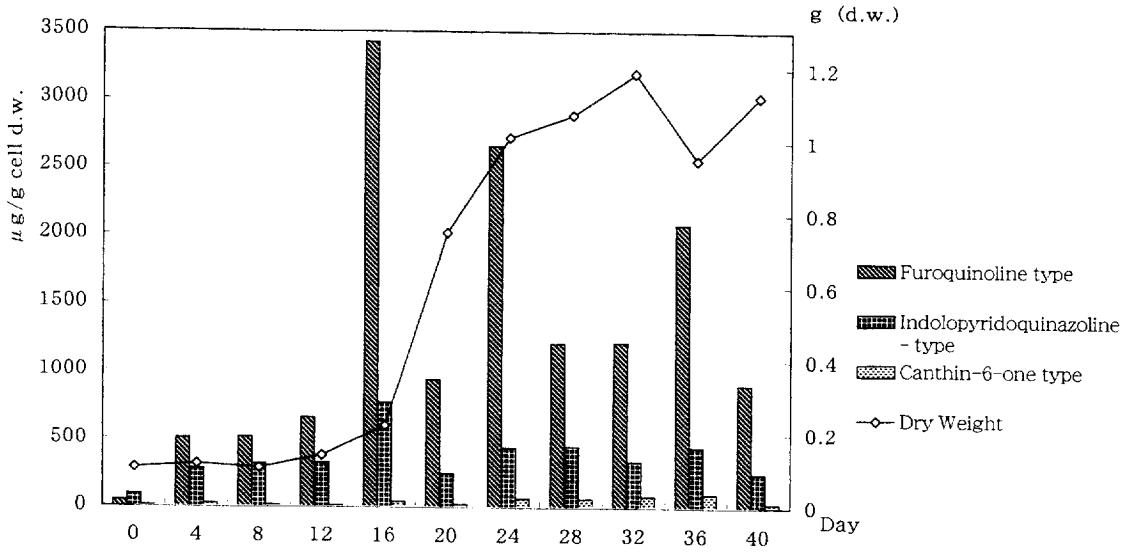


Fig. 2. Time course of alkaloids and dry mass accumulation in NB cell suspension culture *Phellodendron amurense*.

Time course study

A time course study was also undertaken in order to investigate the changes in the composition of various alkaloids over a 40-day-period using cell suspension cultures with NAA and BA. In the suspension cultures, alkaloid productivity was different from the above-mentioned static cultures. Indolopyridoquinazoline type alkaloids, rutaecarpine (**6**) and dehydrorutaecarpine (**7**) were produced in trace amounts, nevertheless, unidentified minor indolopyridoquinazoline type alkaloids were produced at the static culture condition in relatively large amounts instead of the above two alkaloids (**6** and **7**). In the time course study (Fig. 2), the quantities of alkaloids were determined in both the tissue and the medium by HPLC focusing on the furoquinoline type, (dictamnine and skimmianine), the indolopyridoquinazoline type alkaloids, and canthin-6-one (**5**). Furoquinoline type (dictamnine (**8**) and skimmianine (**10**)) and indolopyrido-quinazoline type (unidentified two alkaloids) alkaloids were produced from early to the later stages of the culturing period (40 days). The former was produced in concentrations which ranged from 0.5 to 3.4 mg^{-1} g (0.05–0.3%, dry wt) as the main alkaloid products, with the latter ranging from 0.2 to 0.7 mg^{-1} g (0.02–0.07%, dry wt). On the other hand, canthin-6-one (**5**) was produced in small amount (<0.1 mg^{-1} g, 0.01%, dry wt.) during the latter half of the culturing period (Fig. 2). Berberine (**1**) a main alkaloid of the intact plant was produced, however, only in trace amounts throughout.

The results obtained coincide with the metabolic distance from anthranilic acid being the branch point intermediate in three type alkaloid biosynthetic pathways; furoquinoline type alkaloids originated from anthranilic acid [19] and canthin-6-one originated from tryptophan via anthranilic acid [20], respectively. Furthermore, indolopyridoquinazoline type alkaloids originated from tryptophan plus anthranilic acid [21, 22] (Scheme 1).

The furoquinoline alkaloid pathway, which was not active in the intact plant (*P. amurense*) produced the main alkaloids throughout the culturing period in the suspension cultures. This is interesting not only from a signal transduction perspective, but further, from a chemotaxonomic and phylogenetic point of view.

EXPERIMENTAL

General

^1H and ^{13}C NMR: 500 and 125 MHz (JEOL GSX-500) respectively, room temp, $CDCl_3$, and CD_3OD. Chemical shifts are given in δ (ppm) with (δ 7.26 and 77.0 ppm respectively) as int. standard. Multiplicities for the ^{13}C NMR spectra were determined by DEPT experiments at 90 and 135° and the assignments were determined by HOHAHA, HMQC and HMBC experiments, (Varian Unity-400 spectrometer), MS: 70 eV, direct probe, HRMS measured on a JEOL JMS-SX102A mass spectrometer.

HPLC was carried out on a Waters 600; HPLC column CAPCELL PAK C18 AG120, 5μm, 4.6 × 250 (SHISEIDO); flow rate 0.5 ml min^{-1}; Detection UV 310 nm.

Plant material

Stems and fruits of *Phellodendron amurense* Rup. (Rutaceae) were collected in April 1992 and in October 1996, respectively, at the Medicinal Plant Garden of Science University of Tokyo. The plant material was identified by Dr T. Nakamura, Faculty of Pharmaceutical Sciences, Science University of Tokyo, and

a voucher specimen (No. ph-92-04) was deposited at the herbarium of our Institute.

Callus cultures

Callus tissues from the stems of *Phellodendron amurense* were established in April 1992. Murashige and Skoog medium [23] (minus glycine) (M&S) containing 2,4-D (1 mg l^{-1}–3 mg l^{-1}) and kinetin (KIN) 0.1 mg l^{-1}) as plant growth regulators were used for the induction of callus tissues. The callus tissues were subcultured every 5–6 weeks onto fresh M&S medium containing 2,4-D (1 mg l^{-1}) and KIN (0.1 mg l^{-1}) at 25±1° in the dark.

Alkaloid extraction and isolation

Callus tissues. The *P. amurense* callus tissues were subcultured at 5-week intervals over 7–8 months duration on static MS medium containing 2,4-D (1 mg l^{-1}) with KIN (0.1 mg l^{-1}) as plant growth regulators, and each callus tissue was harvested at 5-week intervals and stored in MeOH for alkaloid investigation until needed. The stored total amounts of callus tissues, fr. wt, 4.5 kg, dry wt 130 g (subcultured during 1993–1994 from establishment of callus tissue), for first investigation, and fr. wt 8.5 kg, dry wt 220 g (subcultured during 1995–1996 from establishment of callus tissue), for a second investigation, were individually extracted with cold MeOH and EtOAc in a Waring blender. The extracts were concd under red. pres. and the residue was partitioned between $CHCl_3$ (2 l) and H_2O (0.3 l) to obtain the organic soluble fr., repeatedly. The $CHCl_3$ solubles were evapd. to dryness and the extracts chromatographed on a column of silica gel (Merck 9385) by gradient elution using $CHCl_3$ with increasing proportions of MeOH. The crude alkaloid mixtures so obtained were purified repeatedly by CC on a SI gel column (Merck 9385) eluted with hexane–EtOAc, to give the following alkaloids: The first investigation: 1: 1.2 mg, 0.0009% 2: 1.0 mg, 0.0008%, 4: 3.5 mg, 0.0027%, **5**: 4.6 mg, 0.0035%, **6**: 3.4 mg, 0.0026%, **7**: 2.9 mg, 0.0022%. The second investigation: 1: 3 mg, 0.0014%, 2: trace amount, 4: 6 mg, 0.003%, **5**: 14 mg, 0.0063%, **6**: 5.5 mg, 0.0025%, **7**: 6 mg, 0.0027%, **8**: 11 mg, 0.005%, 9: 3.6 mg, 0.0016%, 10: 5 mg, 0.0023%).

Fruits. Fruits (2.2 kg, dry wt 412 g) of *Phellodendron amurense* Rup. (Rutaceae) were collected in October 1996, as described above, and repeatedly extracted with cold and hot MeOH (3–4 l). The total extracts were concd under red. pres. and the residue submitted to chromatography on a column of SI gel to afford the crude alkaloid fractions. Further, the crude fractions were separated by using solvent systems with standard samples for TLC analysis (Merck, 1.05715). (a, C_6H_6–EtOAc–CH_3CN = 7:3:0.5; b, MeOH–H_2O–NH_4OH = 8:1:0.5; c, C_6H_6–EtOAc–*n*-Propyl-OH–CH_3OH–NH_2Et = 8:4:2:1:1). Rf value; **6**: 0.55 (a), **1**: 0.48 (c), **2**: 0.358 (c), **4**: 0.19 (b). Further alkaloid **6** was repeatedly purified by CC on a silica gel column and was identified by comparison of its spectral data (^1H, ^{13}C NMR and MS).

7,8-*Dehydrorutaecarpine* **7**

Pale yellow powder, UV λ_{max} (log ε) nm (EtOH) 217 (4.52), 250 (4.49), 281 (4.31), 330 (4.42), 348 (4.38), 368 (4.43), 390 (4.42). IR ν_{max} cm^{-1} (KBr): 1690, 1600, 1475, 1395. EIMS *m/z* (%): 285 (M^+, 100), 256 (13), 229 (5). HRMS (*m/z*): M^+ 285.0896 (calcd for $C_{18}H_{11}ON_3$, requires 285.0902); ^1H NMR ($CDCl_3$): δ 7.33 (1H, *ddd*, *J* = 8,7,1 Hz, 10-H), 7.46 (1H, *ddd*, *J* = 8, 8, 1 Hz, H-3), 7.48 (1H, *ddd*, *J* = 8, 7, 1 Hz, H-11), 7.52 (1H, *dd*, *J* = 8, 1 Hz H-12), 7.55 (1H, *d*, *J* = 8 Hz, H-8), 7.81 (1H, *dd*, *J* = 7, 1 Hz, H-1), 7.82 (1H, *ddd*, *J* = 8, 7, 1 Hz, H-2), 8.02 (1H, *dd*, *J* = 8, 1 Hz, H-9), 8.50 (1H, *dd*, *J* = 8, 1 Hz, H-4), 8.75 (1H, *d*, *J* = 8 Hz, H-7), 10.15 (1H, *br s*, N-H); ^{13}C NMR ($CDCl_3$): δ 107.7 (*d*, C-8), 112.3 (*d*, C-12), 120.9 (*s*, C-8a), 118.8 (*d*, 7-C), 116.7 (*s*, C-4a), 120.9 (*d*, C-9), 121.3 (*d*, C-10), 122.5 (*s*, C-9a), 127.4 (*d*, C-11), 124.8 (*d*, C-3), 126.0 (*d*, C-1), 127.7 (*d*, C-4), 134.6 (*s*, C-13a), 134.9 (*d*, C-2), 139.5 (*s*, C-12a), 140.1 (*s*, C-14a), 147.5 (*s*, C-1a), 159.2 (*s*, C=O), (*d* = , CH, *t* = CH_2, *q* = CH_3 from DEPT experiments).

Time course study

Suspension culture. Phelodendron amurense cell suspension cultures were generated from 30- to 40-day old static cultures (agar medium) grown at 25±1 C on MS containing 1.0 mg l^{-1} NAA (1-naphthalene acetic acid) and 0.1 mg l^{-1} BA (6-benzylamino purine). Approximately 8 g of callus was transferred to 500 ml Erlenmeyer flasks containing 100 ml of liquid MS media and cultured every 4 weeks on rotary shaker (120 rpm min^{-1}).

Ten ml aliquots of the cell suspension were added to Erlenmeyer flasks (500 ml) containing 100 ml of MS with hormone (NAA: 1.0 mg l^{-1} and BA: 0.1 mg l^{-1}). After inoculation, the flasks were placed onto a rotary shaker (125 rpm/min.) at 25±1° C and allowed to culture for 4, 8, 12, 16, 20, 24, 28, 32, 36 and 40 days.

Cells were harvested at each time-point by suction filtration and extracted by sonication in MeOH (10 ml) for 30 min at room temp. and concentrated *in vacuo*. The callus MeOH extract and the media were separately extracted with $CHCl_3$ and the amount of alkaloids in each determined by HPLC.

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