A novel root inducer, 4-(3-indolyl)-4-butanolide (IBL), is formed at an early stage in Bupleurum falcatum L. root cultures containing indole-3-butyric acid (IBA)

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Abstract We found a novel indolebutyric acid (IBA) derivative, 4-(3-indolyl)butanolide (IBL), produced at an early stage during Bupleurum falcatum L. (known as Saiko in Japan) root culturing. Another IBA derivative, 4-[3-(2-oxo-indolyl)]-butyric acid, was also detected, but this compound appeared to have an abiotic origin. Synthetic IBL induced rooting in cultured roots of B. falcatum and induced rooting in cuttings of Vigna radiata. The root-inducing characteristics of IBA and IBL were different. IBA induced considerable thickening of the original roots of B. falcatum, because of marked cell expansion and division in the cortical tissue, before new roots emerged, a typical response to auxin. IBL, on the other hand, did not cause cell expansion, but it induced cell division in the cortical tissue as strongly as IBA did. IBL has the potential to become an excellent, industrially used root inducer, and it serves as valuable tool for research on root induction, because of its unique root-inducing activity.

Key words: Bupleurum falcatum L., IBA, IBL, rooting, stress.
emerged. Furthermore, drought stress caused a 5-fold increase in IBA-induced rooting (Yokoyama et al. 2003). We previously observed that a specific, oxidized linolenic acid derivative (KODA), which is induced under stress conditions, was involved in flower induction in *Lemma paucicostata* (Yokoyama et al. 2000). For this reason, we hypothesized that IBA is oxidized when the cells are exposed to an oxidative environment, such as that involved in rooting. Here, we report the identification of IBL, a novel metabolite of IBA, which was detected outside the cells during the earliest stage of the culture, and we describe the characteristics of IBL by comparing it to IBA.

**Materials and methods**

**Culturing of *B. falcatum* roots and rooting tests on cultured roots with IBL**

The *B. falcatum* strain used throughout this study was described in a previous study (Kusakari et al. 2000), as was the cultivation procedure for adventitious roots of *B. falcatum* (Kusakari et al. 2000). The roots of seedlings, which had been germinated from sterilized seeds and grown aseptically, were detached and cultured on LS (Linsmaier and Skoog 1965) medium, solidified with 0.2% gellan gum (San-Ei Gen F.F.I. Osaka, Japan). The medium was supplemented with 0.1 mg l$^{-1}$ IBA, as potassium salt (Sigma, MO, USA). The roots swelled to some extent, and generated many adventitious roots, which were cultured in liquid B5 medium (Gamborg et al. 1968) supplemented with 8 mg l$^{-1}$ (40 μM) IBA. The clone that grew fastest and produced the largest amounts of saikosaponin-a and -d, was selected and maintained. Subculturing was done by transferring 2 g of the roots to 400 ml of fresh B5 medium in a 1000-ml flask containing 5 mg l$^{-1}$ IBA. The roots were incubated for 3 weeks on a gyratory shaker (105 rpm) at 23°C in the dark. After these 3 weeks, the medium was replaced by medium without IBA, and the roots were cultured for another 6 weeks. For isolating the compounds released into the medium, the roots that emerged from both the hypocotyls and the epicotyls were counted.

**Microscopic observation of cortical cells in *Bupleurum falcatum* roots**

Sections of *B. falcatum* were prepared as described previously (Ayano et al. 2005). Roots of *B. falcatum* cultured in B5 medium with or without IBA or IBL (4 or 8 mg l$^{-1}$) were fixed in FAA solution (formaldehyde and acetic acid at 5% each in 50% ethanol). The medium parts (2.5–3.0 mm) were isolated, dehydrated in Histresin, embedded in Histresin:hardener (15:1), and sectioned at 3.0 μm. Staining was performed with 0.3% toluidine blue and 0.2% sodium tetraborate at 60°C for 6 min, followed by 2% orange G and 5% tannic acid with phenol for 1 min, 5% tannic acid for 1 min, and 0.2% ferrous ammonium sulfate for 1 min.

**Statistical analysis**

The significance of differences was determined using the $t$ test. Significant differences are indicated as follows: * $P<0.05$, ** $P<0.01$, *** $P<0.005$, **** $P<0.0001$.

**Analyses of substances 1 (IBL) and 2**

Substances 1 (IBL) and 2 were isolated by chloroform extraction from the culture medium or roots. Vacuum drying resulted in a powder, which was dissolved in methanol and subjected to HPLC with a UV (214 nm) detector and a Capcell Pak C$_{18}$ column (250 mm×4.6 mm internal diameter (I.D.), Shiseido, Tokyo, Japan). A solution of 30% acetonitrile [0.1% trifluoroacetic acid (TFA)] was used for elution at 1 ml min$^{-1}$. IBA, 1 (IBL) and 2 were eluted at retention times of ca. 16.5, 11, and 5.5 min, respectively.

In order to isolate 1 (IBL), 8 g of roots of *B. falcatum* were chopped with a sterilized blade into ca. 1 cm fragments and transferred to the B5 medium (400 ml in 1000-ml flask) containing 8 mg l$^{-1}$ IBA, since this procedure stimulated formation of the compounds. The flasks were incubated on a shaker for 18 h. The medium from all the flasks (121 in all) was extracted using chloroform. After removal of the solvent in vacuo, the residue was dissolved in methanol and subjected to HPLC under the conditions described above, except for the column diameter (10 mm I.D.) and the flow rate (4 ml min$^{-1}$). The yield was 12 mg of IBL and 3 mg of compound 2.

Compound 2 coincided with the peak of the oxidation product of IBA with m-chloroperbenzoic acid.

**Chemical synthesis of IBL**

IBL was also synthesized chemically to verify the structure. An indole anion, produced from indole and ethylmagnesium bromide, reacted with succinic anhydride to yield 4-(3-indolyl)-1-oxobutyric acid. The oxo group was reduced with NaBH$_4$, with plastic wrap, and cultivated at 22°C under continuous light. When the seedlings grew as high as 10 cm, the cotyledons were removed, and the hypocotyls were cut to a length of 2 cm. The cuttings were placed in 40 ml of test solution containing IBA or IBL in 50-ml glass vials. For this experiment, IBA and IBL were dissolved in dimethyl sulfoxide (DMSO) at a 1000-fold concentration, and diluted to give the required final concentration. Six cut seedlings were put in separate vials and incubated at 22°C under continuous light (50 μE s$^{-1}$ m$^{-2}$). The roots that emerged from both the hypocotyls and the epicotyls were counted.
resulting in IBL. The NMR spectrum of chemically synthesized IBL was identical to that of compound 1 from the roots. Chemically synthesized IBL was used in the experiments. Natural IBL is stereoisomeric, with an asymmetric carbon at the 1’ position, but the chemically synthesized product was racemic.

4-(3-indolyl)butanolide (1)
Pale red needles, UV $\lambda_{\text{max}}$ nm: 270. IR (KBr, cm$^{-1}$): 1750. HR/FAB-MS (Negative): 200.2250, Calcd. 200.2244. $^1$H NMR (CDCl$_3$, $\delta$): 2.73 (m, 2-H2), 2.60, 2.75 (m, 3’-H2), 5.89 (dd, $J=7.0, 8.3$, 1’-H), 7.04 (dd, $J=7.0, 7.8$, 5-H), 7.14 (dd, $J=8.3, 7.0$, 6-H), 7.33 (s, 2-H), 7.37 (d, $J=8.3, 7.4$, 3-H), 7.59 (d, $J=7.8, 4$-H), 8.30 (br s, 1-H). $^{13}$C NMR (CDCl$_3$, $\delta$): 29.5 (C-2), 30.4 (C-3), 79.2 (C-1), 112.6 (C-7), 115.6 (C-3), 119.9 (C-5), 120.1 (C-4), 122.5 (C-6), 124.2 (C-2), 127.1 (C-3b), 138.5 (C-2b), 180.3 (C-4’). Positive FAB-MS: $m/z$ 202 (M+H)$^+$. 

4-[3-(2-Oxo-indolyl)]butyric acid (2)
Colorless powder. HR/FAB-MS (Negative): 218.2405, Calcd. 218.2398. $^1$H NMR (DMSO-d$_6$, $\delta$): 1.48 (m, 2-H2), 1.78, 1.85 (m, 1’-H2), 2.19 (dd, 3’-H2), 3.42 (dd, 3-H), 6.82 (d, 7-H), 6.95 (dd, 5-H), 7.17 (dd, 6-H), 7.23 (d, 4-H), 10.35 (s, 1-H), 11.95 (brs, 4’-OH). $^{13}$C NMR (DMSO-d$_6$, $\delta$): 20.85 (C-2), 29.3 (C-1’), 33.5 (C-3’), 44.8 (C-3), 109.1 (C-7), 121.1 (C-5), 123.9 (C-4), 127.5 (C-6), 129.5 (C-3b), 142.6 (C-2b), 174.0 (C-2), 178.6 (C-4’). Positive FAB-MS: $m/z$ 220 (M+H)$^+$. 242 (M+Na)$^+$. 

Results

IBL is generated from IBA at an early stage of the root culture

When roots of Saiko (B. falcatum L.) were cultured in B5 medium containing IBA (40 $\mu$M), vigorous rooting occurred after approximately 2 weeks. During the initial period after subculturing, when the roots are expected to be exposed to the highest stress level (Yokoyama et al. 2003), HPLC analysis of the medium revealed 2 novel peaks, in addition to that of IBA. One of them was an oxidized derivative of IBA, generated abiotically, because it was formed even when IBA alone was incubated overnight (without roots). Its chemical structure is presumed to be oxindolebutyric acid (4-[3-(2-oxo-indolyl)]butyric acid) (no. 2 in Figure 1), based on its co-elution in HPLC with the oxidation product of IBA with m-chloroperbenzoic acid. To isolate the other compound, we chopped the roots before adding them to the medium, because its production increased at least 1.5-fold by this treatment. The compound was extracted by conventional means, and purified by HPLC. Its structure was identified as 4-(3-indolyl)butanolide (IBL) (no. 1 in Figure 1). This is a novel compound, and it was synthesized chemically to confirm its structure. The NMR spectrum of the chemically synthesized product was identical to that of IBL formed in the root culture (data not shown). IBL is expected to be formed via 4-hydroxy-4-(3-indolyl)butyric acid (Figure 1).

IBA was added to the medium together with the starter roots, and the concentrations of IBA and IBL in the medium were recorded (Figure 2A). The IBA concentration declined rapidly, and on day 4, IBA was no longer detectable in the medium or the tissue. The IBL concentration in the medium was approximately 5 $\mu$M at day 1, and this remained constant until at least day 4. However, it was no longer detectable after 8 days. The concentrations of IBA and IBL in the root tissue were

Figure 1. The putative pathway to formation of IBL (1) and oxindolebutyric acid (2) from indolebutyric acid (IBA).

Figure 2. IBA and IBL concentrations in the medium. IBA (40 $\mu$M) was added on day 0 or day 7 of a B. falcatum root culture. (A) The IBA (■) and IBL (●) concentrations in the medium were measured after addition of IBA at day 0. (B) The solid line shows the concentrations of IBA (■) and IBL (●) after IBA was added on day 0. The dotted line shows the concentrations when IBA was added on day 7. Three separate experiments yielded similar results.
only roughly 1% of those in the medium. When IBA was added 7 days after subculturing, on the other hand, the IBL concentration was almost below the detection limit, while the IBA concentration decreased at the same rate (Figure 2B).

**Effect of IBL on rooting**

Chemically-synthesized (racemic) IBL was used for testing its root induction activity on *B. falcatum* roots. The root induction activity of 20 μM IBL was as strong as that of IBA at the same concentration. However, the root induction response to IBA continued to increase up to a concentration of 160 μM, while the response to IBL reached a maximum at 40 μM. Beyond that, the response decreased with increasing concentrations. At 160 μM IBL, no root induction was observed (Figure 3A). We also tested the root induction activity on *V. radiata* cuttings, which is one of the standards for physiological rooting test. In *V. radiata* cuttings, the activities of IBL and IBA were equal at 100 μM, while at 10 μM, the activity of IBL was weaker than that of IBA (Figure 3C).

The morphological changes in the transplanted roots exposed to IBL or to IBA were markedly different. IBA caused swelling of the roots over the 2 days prior to the generation of new roots, which occurred after 2 weeks of incubation. Similar swelling was observed for hypocotyls of *V. radiata* cuttings. In contrast, IBL caused little swelling of *B. falcatum* roots or *V. radiata* cuttings (see Figure 3B, D). We prepared cross and longitudinal sections of *B. falcatum* roots to investigate the histological changes. As can be seen in Figure 4, IBA caused cortical cells to expand, while in control roots, there were no changes in either the cortical cells or the diameter of the roots. With IBA (20 and 40 μM), the diameter of the roots increased gradually up to 12 days (Figure 5A), when the primordia of newly emerged roots had been formed in pericycles. The expansion of cortical cells reached its maximum by day 5 (Figure 5C), and this preceded the maximum diameter of the roots. We also observed many phragmoplasts in the cortical cells in the experiments with IBA, indicating that cell division had occurred (Figure 4). The number of phragmoplasts increased concomitantly with the root thickening (Figure 5A, B). Thus, IBA-induced thickening of the root was a result of the expansion of the cortical cells, which was followed by cell division. IBL at 40 μM did not induce cell expansion in the cortical layer, but did induce cell division, causing the diameter of the roots to increase (Figure 5). IBL also caused a distinct increase in cell division at 20 μM, although this concentration was not associated with apparent root thickening (Figure 5A, B). These changes in cortical cell division or expansion were not observed in control roots (Figure 5B, C). Similarly, IBL failed to cause any distinct swelling of the hypocotyls in *V. radiata* cuttings, while 100 μM IBA (Figure 3D) or IAA (data not shown) caused a significant swelling of the hypocotyls. Many new roots appeared in the epidermal gaps produced by the expanded cortical tissue.

The rooting rate of *B. falcatum* roots in the presence of IBA is greatly affected by the timing of IBA addition (Yokoyama et al. 2003). The rooting response to IBA was compared to the response to IBL when they were added at different time points during root culturing (Figure 6). The rooting rate of transplanted roots of *B. falcatum* decreased when the addition was delayed. This was true with both IBA and IBL, although the decrease was less severe in IBL than in IBA (Figure 6).

**Discussion**

It appears that IBL is produced under stress conditions, because it was formed at the initial stage of the root culture of *B. falcatum*. However IBL production was negligible when IBA was applied to roots that had been pre-cultured for 1 week in the absence of IBA (Figure 2B). We reported previously that the H₂O₂ concentration in the roots increases at an early stage during root culturing. It then declines sharply until new roots emerge, 2 weeks later (Yokoyama et al. 2003). Sugars are specific scavengers of hydroxyl radicals (Asada and Kiso 1973), and sucrose suppresses the lateral root formation of cultured roots of *B. falcatum* (Kusakari et al. 2000). The hypothetical precursor of IBL, i.e. the hydroxide form (Figure 1), might be generated under such oxidative conditions.

The importance of IBL for root induction with IBA remains to be established. IBL is generated at the early stage of the culture while IBA is present. Moreover, the concentration of IBL is relatively low. Therefore, if IBL plays a role in root induction, it is speculated that its activity is not dominant. As a novel root inducer, however, IBL is promising. Although the effect on rooting in *B. falcatum* cultured roots was surely weaker than IBA, it is important to keep in mind that the IBL used was a racemic compound on the fourth carbon of butanolide. Natural IBL produced by *B. falcatum* root tissue is optically active, and the biological activities of the isomers remain to be examined. Moreover, the potency of IBL to induce rooting in *V. radiata* cuttings was almost the same as that of IBA (Figure 3). For two other plant species, IBL has a potential advantage over IBA. Yano and Yokoyama (unpublished data) studied the effect of IBL and other auxins on the propagation of cuttings of *Michelia compressa* (Maxim.) Sargent var. *Formosa* Kanehira. Root induction is difficult in this plant species, both with and without CO₂ enrichment.

The rooting rate of the cuttings was highest in 10 ppm IBL with CO₂ enrichment. In the other study it was reported that in *Paraserianthes falcataria*, which is a
Figure 3. The effects of IBA and IBL (I) on rooting in cultured roots of *B. falcatum* or cuttings of *V. radiata*. (A) In *B. falcatum*, the new roots were counted after 3 weeks, in a 2 cm section of the centre part of the transplanted roots, at different concentrations of IBA (gray bar) or IBL (pink bar). (B) Images showing newly-emerged roots on the transplanted roots. Note the swelling of the transplanted roots in the presence of IBA and the absence of swelling in the presence of IBL. (C and D) *V. radiata* seedlings were dipped in aqueous solution with different concentrations of IBA or IBL, after removal of the cotyledons, and they were incubated in continuous light. (C) Roots induced by IBA (open bar) or IBL (hatched bar) were counted after 3 weeks. (D) Images showing the seedlings with roots at the positions where the cotyledons were removed (arrows). Values are given as mean±SD of root number (n=6–10). Each experiment was repeated at least twice with similar results. Typical results are shown in this figure. ****P<0.0001. The bar indicates 1 cm.

Figure 4. Cross-section of cultured roots of *B. falcatum* L. The roots were cultured in B5 medium (control), or in the same medium containing IBA (40 μM) or IBL (40 μM), and harvested at day 0, 5, or 12. After that, the roots were fixed and prepared for cross-sections. Representative sections are shown. The bar indicates 100 μm.
fast-growing tropical tree, rooting was not promoted with 0.01 mM IBA, and was even severely inhibited at concentrations of 0.1 mM and above. In contrast, IBL improved the rooting rate, and increased the root mass up to 0.5 mM, and the increase was concentration dependent (Yokoyama and Nakamura 2007). This activity of IBL does not have the adverse effects that are commonly observed at higher concentrations of auxins, like IBA. This may be attributed to the fact that IBL does not induce cortical cell expansion (Figures 4, 5). IBL seems to specialize on root differentiation.

It is important to mention that IBL failed to activate rooting in Arabidopsis thaliana (data not shown). In A. thaliana, IBA essentially elicits rooting activity as a result of it being converted to IAA by β-oxidation in peroxisomes (Woodward and Bartel 2005). This may imply that an IBL-dependent pathway exists, and that IBL does not just act as an analogue of IBA or IAA. Further study on this unique rooting activity of IBL is currently in progress, and we expect to develop it into a new type of root inducer.

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