Oxylipins are acyclic or cyclic oxidation products that are formed during the catabolism of fatty acids. Among them, jasmonic acid (JA) has been reported to be involved in plant flowering. It has been shown that mRNA of allene oxide cyclase, which catalyses the conversion of allene oxide to cis-(12R)-12-oxophytodienoic acid (OPDA), a crucial step in the biosynthesis of JA, accumulates in flower buds of *Licopersicon esculentum* (tomato) (Hause et al. 2000). Constitutive expression of allene oxide cyclase in tomato caused a dramatic elevation of the levels of JA and related oxylipins in flower organs, but not in leaves (Miersch et al. 2004). JA enhanced flower induction in *Lemna minor* under long-day conditions, but prevented flower induction at higher concentration under the same conditions (Krajncic et al. 2006).

Ives and Posner (1982) showed that in ammonium-free medium, propranolol, a β-adrenergic blocking agent, inhibited flowering of *Lemna paucicostata* strain 441, and this inhibition was completely overcome by epinephrine, l-epinephrine, l-norepinephrine, and l-isoproterenol, which substantially promoted flowering under short-day conditions (Khurana et al. 1987).

The aqueous homogenate of *L. paucicostata*, strain 441, induces flowering of *L. paucicostata*, strain 151 (P151) (Takimoto et al. 1989; Takimoto and Kaihara. 1990). Two factors are involved in this flower-inducing activity, one being (−)-norepinephrine (NE) (detected in the supernatant after centrifugation of the plant homogenate), and the other being the α-ketol type oxylipin, 9-hydroxy-10-oxo-12(Z),15(Z)-octadecadienoic acid (KODA), which is induced by stresses, such as drought, heat and osmotic stress (Takimoto et al, 1994; Yokoyama et al, 2000). Neither alone was active, but their mixture was active after incubation (Yokoyama et al. 2000). We previously isolated two major reaction products of KODA and NE, and determined their absolute chemical structures as (9R)-11-{[(2R,8S,10'S,11'S)-2',8'-dihydroxy-7'-oxo-11'-[(Z)-2-pentenyl]1'-9'-oxa-4'-azatricyclo[6.3.1.01,5]dodec-5'-en-10'-yl]-9-hydroxy-10-oxoundecanoic acid (FN1) and its C-9 epimer (FN2). FN1 showed a strong flower-inducing activity per se.

**Abstract** The reaction of 9-hydroxy-10-oxo-12(Z),15(Z)-octadecadienoic acid (KODA) and catecholamine in water (pH 8.0) in vitro generates many products, and we have shown that the major adduct FN1 among the reactants of KODA and norepinephrine is a potent inducer of floral differentiation in *Lemna (L.) paucicostata* 151 [Yokoyama et al. (2000) Plant Cell Physiol 41: 110–113, Yamaguchi et al. (2001) ibid 42: 1201–1209]. Since such reactants may be involved in flower induction in vivo, it is of interest to elucidate the structure-activity relationships among the reactants to throw light on the nature of the putative active site. Here, we report two other adducts, designated as FN7 and FN10. They are structurally similar to FN1, having a tricyclic structure with 12-cis-olefin conjugated with noradrenochrome. FN10 was a more potent inducer than FN1. These results are consistent with the hypothesis that tricyclic KODA/norepinephrine adducts play an important role in flower-induction in *L. paucicostata*, strain 151.

**Key words:** Catecholamine, flower induction, FN, KODA, *Lemna paucicostata*. 

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Identification of reaction products of KODA and norepinephrine that strongly induce flowering in Lemna

Materials and methods

Culture of Lemna paucicostata

Flower-inducing activity was measured as described previously (Takimoto et al. 1994), using Lemna paucicostata (P151). The plants were subcultured aseptically on half-strength Hutner’s medium (Hutner 1953) containing 1% sucrose, under continuous light provided by cool-white fluorescent tubes (20 W m⁻²) at 25°C. For the assay, the plants were cultured initially in the assay medium (one-tenth strength E medium containing 1 μM benzyladenine, but no sucrose), and then in the same medium, but in the presence of a reactant of KODA and NE, or purified FN7 or FN10.

Analyses of FN7 and FN10

KODA was prepared using lipoygenase from rice germ and allene oxide synthase from flax seed (Yokoyama et al. 2000). In order to obtain the reactants, KODA (6.45 mmol) in distilled water (3.3 L) was added to NE (6.45 mmol in 645 mL of water) in 1 M Tris–HCl buffer (pH 8.0, 320 mL) and the solution was incubated for 160 hr at 25°C. The reaction mixture was analyzed by means of HPLC with a UV (210 nm) detector and a CAPCELLPAK C_18 column (250 mm×4.6 mm I.D., Shiseido, Tokyo, Japan). Elution was done with 50% acetonitrile [0.1% trifluoroacetic acid (TFA)] at 1 mL/min. The following instruments were used to obtain physical data: specific rotations, SEPA-300-3000 digital polarimeter (L=5 cm, Horiba, Kyoto, Japan); UV spectra, UV-2200 spectrometer (Shimadzu, Kyoto, Japan); IR spectra, FT/IR-5300 spectrometer (Jasco, Tokyo, Japan); fast atom bombardment mass spectrometry (FAB-MS) and high-resolution FAB-MS, MAT95Q mass spectrometer (Finnigan MAT, MA, U.S.A.); 1H-nuclear magnetic resonance (NMR) spectra, ECP-400 (400 MHz) spectrometer (JEOL, Tokyo, Japan); 13C-NMR spectra, ECP-400 (100 MHz) spectrometer (JEOL); HPLC, LC100 system (Yokogawa, Tokyo, Japan) or NANOSPACE SI-1 chromatograph (Shiseido).

Physico-chemical properties

FN7-1:

FAB-MS: m/z 494 [M+H]^+ (positive), m/z 492 [M–H]^- (negative), HR-FAB-MS m/z 494.2751 (found), (–0.3 mmu) 494.2754 (calc. for C_{13}H_{16}O_{10}N) [M+H]^+; (positive). UV Jmax (MeOH, nm) 295 nm.

1H-NMR (400 MHz, DMSO-d6): δ 0.93 (3H, t, J=7.3, 5s-H), 1.20–1.45 (8H, 4.5,6,7-H), 1.25, 1.46 (both 1H, m, 3-H), 1.40, 1.89 (both 1H, m, 8-H), 1.50 (1H, m, 11s-H), 1.94, 1.98 (both 1H, m, 12-H), 2.09 (2H, m, 4-H), 2.12 (2H, t-like, J=7.3, 2-H), 2.13, 2.50 (both 1H, m, 1-H), 3.00 (1H, m, 9-H), 3.18 (1H, m), 3.63 (1H, dd, J=3.4, 12.0, 3-H), 4.08 (1H, dd, J=4.3, 7.8, 9-H), 4.12 (1H, d, J=3.4, 2-H), 4.23 (1H, m, 10-H), 4.98 (1H, s, 6s-H), 5.27 (1H, m, 2s-H), 5.30 (1H, m, 3s-H), 8.11 (1H, s, NH).

13C-NMR (100 MHz, DMSO-d6) δ: 13.9 (C-5s), 20.2 (C-4s), 22.6 (C-1s), 24.5 (C-3), 25.0 (C-7), 28.5, 28.7, 28.8 (C-4,5,6), 31.7 (C-8), 33.7 (C-2), 35.5 (C-12), 42.0 (C-11), 53.6 (C-3s), 55.3 (C-9s), 55.4 (C-1s), 67.3 (C-10s), 71.0 (C-2s), 74.4 (C-8s), 77.9 (C-9), 91.9 (C-6s), 130.2 (C-3s), 131.0 (C-2s), 173.1 (C-5s), 174.5 (C-1s), 192.0 (C-7s), 211.4 (C-10).

FN7-2:

FAB-MS: m/z 494 [M+H]^+ (positive), m/z 492 [M–H]^- (negative), HR-FAB-MS m/z 494.2746 (found), (–0.8 mmu) 494.2754 (calc. for C_{13}H_{16}O_{10}N) [M+H]^+; (positive). UV Jmax (MeOH, nm) 295 nm.

1H-NMR (400 MHz, DMSO-d6): δ 0.93 (3H, t, J=7.3, 5s-H), 1.20–1.45 (8H, 4.5,6,7-H), 1.30, 1.50 (both 1H, m, 3-H), 1.48, 1.88 (both 1H, m, 8-H), 1.60 (1H, m, 11s-H), 1.94, 1.99 (both 1H, m, 12s-H), 2.09 (2H, m, 4-H), 2.18 (2H, t-like, J=7.3, 2-H), 2.10, 2.50 (both 1H, m, 1-H), 3.27 (1H, m, 9-H), 3.34 (1H, m), 3.62 (1H, dd, J=3.4, 12.0) (3-H), 3.79(1H, dd, J=4.3, 7.8, 9-H), 4.13 (1H, d, J=3.4, 2-H), 4.19 (1H, m, 10-H), 4.99 (1H, s, 6s-H), 5.28 (1H, m, 2s-H), 5.30 (1H, m, 3s-H), 8.08 (1H, s, NH).

13C-NMR (100 MHz, DMSO-d6) δ: 13.9 (C-5s), 20.2 (C-4s), 22.6 (C-1s), 24.5 (C-3), 25.0 (C-7), 28.5, 28.7, 28.8 (C-4,5,6), 31.7 (C-8), 33.7 (C-2), 35.5 (C-12), 42.0 (C-11), 53.6 (C-3s), 55.3 (C-9s), 55.4 (C-1s), 67.3 (C-10s), 71.0 (C-2s), 74.4 (C-8s), 77.9 (C-9), 91.9 (C-6s), 130.2 (C-3s), 131.0 (C-2s), 173.1 (C-5s), 174.5 (C-1s), 192.0 (C-7s), 211.4 (C-10).

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Results

Isolation of FN7 and FN10

The HPLC profile of the reaction products of KODA and NE is shown in Figure 1. Fractions were collected at 5-min intervals and assayed for flower-inducing activity; every fraction showed distinct activity (Yamaguchi et al. 2001). We planned to isolate the components in peaks FN6, FN7, and FN10, in addition to FN1, because they seemed to show the highest activities. The whole reaction mixture was lyophilized and divided into 5 fractions by chromatography with on a reverse-phase silica gel column (Chromatorex DM1020T, Fuji Silysia Chemical, Aichi, Japan) (Fr. 1: 100% H2O, Fr. 2: 20% MeOH, Fr. 3: 40% MeOH, Fr. 4: 60% MeOH, Fr. 5: 100% MeOH). Fr. 3 (2 g) was further subjected to normal-phase chromatography (Silica gel 60, Kanto Chemical, Tokyo, Japan) [CHCl3-methanol-H2O (8:3:1 lower layer)] and then purified by HPLC on an ODS column [CAPCELLPAK C18, 250 mm×10 mm I.D. with 25% CH3CN (0.1% TFA), Shiseido, Tokyo, Japan] to give FN10 (8.1 mg). The fraction containing FN7 was further separated by HPLC on an ODS column [identical with the one described above, except for the use of 30% CH3CN (0.1% TFA)], affording two epimers, FN7-1 (5.4 mg) and FN7-2 (1.6 mg). FN7-1 showed molecular-related ion peaks at \(m/z\) 506 (M-H) and 508 (M+H) in negative and positive FAB-MS, respectively, and its molecular formula was established as C26H37O9N through high-resolution MS analysis of (M-H). The UV spectrum was similar to that of FN1, showing an absorption maximum at 295 nm. Comparison of the 1H- and 13C-NMR spectra of FN7-1 with those of FN1 revealed that the C-9' carbon is that of a methine group (δ3.08, δ55.3) instead of methylene (C-11 of FN1). The correlation between C-8' and C-9' suggested that the pyran ring of FN1 has been replaced with a cyclohexane ring. The coupling constant between 9'-H and 10'-H was \(J=11.0\) Hz, and the 10'-H signal was a double doublet. In addition, NOE was observed between 9'-H and 12'-H, 3'-H and 11'-H. Thus, the structure of FN7-1 was presumed to be as shown in Figure 2. In order to identify the epimers of FN7, 9R- and 9S-KODA, isolated on a Chiralcel OD-R column (Daicel Chemical Industries, Ltd, Tokyo) (Yamaguchi et al. 2001), were separately reacted with NE. Reverse-phase HPLC (30% CH3CN, 0.1% TFA) comparison of the products with the epimers of FN7 indicated that FN7-1 is the 9R-epimer and FN7-2 is the 9S-epimer. FN10 showed the same molecular weight as FN1 (493), with molecular-related ion peaks at \(m/z\) 492 (M-H) and 494 (M+H). The molecular formula was C26H39NO8, based on high-resolution MS analysis. The UV spectrum was similar to that of FN1, with an absorption maximum at 293 nm. The \(^1\)H and \(^13\)C-NMR spectra indicated that FN10 is a mixture of components with the same molecular formula in a ratio of about 1:1. The acetylation product of FN10 showed 2 peaks upon HPLC. These data suggested that FN10 is an enantiomeric mixture. The \(^1\)H and \(^13\)C-NMR spectra of FN10 were very similar to those of FN1 and FN2, except...
for the signals of the ring structure. The NOESY spectrum was also analyzed, and finally, FN10-1 and -2 were concluded to be isomers of FN1 and FN2, with the tricyclic part linked to the C18 chain at a different position (Figure 2). We could not isolate FN6 because of its instability: the HPLC profile of the peak changed readily during the purification process, although the FN6-containing fraction showed stronger activity than FN7 or FN10, and FN6 also seemed to be composed of stereoisomers, judging from the HPLC profile.

Biological activities of FN7 and FN10
The flower-inducing activities of FN7-1, FN7-2 and FN10 (as an epimeric mixture) were examined. FN7-1 and FN10 were active, while FN7-2 (9S-epimer) showed no activity, like FN2 (9S-epimer) (Figure 3). At lower concentration, FN10 had the strongest activity of them all (including FN1). The activities were in the order of FN10 > FN1 > FN7-1.

Discussion
KODA is an oxylipin that is common in green plants (Vick and Zimmerman. 1987). It is formed from linolenic acid by 9-specific lipoxygenase; this is different from the pathway to jasmonic acid, which involves a 13-specific lipoxygenase (Howe and Schilmiller. 2002). The total structure of KODA, including the α-ketol, carboxylic acid and two cis-olefin moieties, is strictly required for flower-inducing activity in Lemna (Yokoyama et al. 2000). In this report, we showed that two other reactants of KODA and norepinephrine, which also exhibit flower-inducing activity, retain the essential structure of KODA. The structures of FN7 and FN10 resemble that of FN1, but, in FN7, the oxide of NE, noradrenochrome, does not bond at the C12 double bond of KODA. As KODA is rather unstable, especially under alkaline conditions, our present findings are consistent with the idea that the KODA structure is required for flower-inducing activity and that its degradation results in loss of the activity.

Cakehocholamine structure is also important for flower-inducing activity in Lemna (Yamaguchi et al. 2001). Cakehocholamines are widespread in the plant kingdom, as well as in mammals (Smith 1977). Cakehocholamine was confirmed to be involved in flowering in Lemna in an experiment using a cakehocholamine pathway-blocking agent, propranolol (Khurana and Tamot 1987). Cakehocholamine also has other activities, such as ethylene production, nitrogen fixation, and defense against herbivores and injury (Dai et al. 1993; Kuklin and Conger 1995). Recently, it was established that cakehocholamine synthesis is activated in potato leaves under stress, through induction of gene expression of synthetic enzymes, such as tyrosine decarboxylase, when the plant is exposed to high salt, drought or UV irradiation (Swiedrych et al. 2004). Cakehocholamines are proposed to exert their activities through DoH-CB proteins, which retain the cytochrome b561 domain (CB) and the cakehocholamine-binding regulatory domain of dopamine-β-hydroxylase (DoH) (Verelst and Asard 2004). Cytochrome b561 catalyses ascorbate-mediated trans-membrane electron transport, and hence may be involved in ascorbate regeneration (Wakefield et al. 1986; Asard et al. 1998). These facts imply that cakehocholamines may be involved in ascorbate regeneration. We have observed that cakehocholamines, such as epinephrine and norepinephrine, and not only KODA, are released when Lemna is exposed to drought stress (unpublished data). Thus, both KODA and cakehocholamine appear to be stress-induced substances that react to generate compounds that directly induce flowering in L. paucicostata. We have tried to detect adducts such as FN1, FN7 and FN10 in L. paucicostata by means of LC-MS analysis, but so far without success. Based on the observed activity of the reactants of KODA and NE, the effective concentrations of these adducts in vivo could be at picomolar levels, and this may be the...
reason why they were not detected. However, we can not rule out the possibility that compounds other than FN1, FN7 or FN10 are the active agents in L. paucicostat a in vivo. Other active adducts may be generated by the reactions of other catecholamines, such as dopamine and epinephrine (Yamaguchi et al. 2001). Structure-activity analyses of many adducts reveal the essential structural requirements for flowering-inducing activity in L. paucicostata in nature, and thereby provide clues as to the mechanism involved and the development of a flower-regulating agent.

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References


Smith YA (1977) Phenylethylamine and related compounds in plants. Phytochem 16: 9–18


Yokoyama M (2005) Roles of 9,10-α-ketol linolenic acid (KODA), one of the oxylipins formed with 9-lipoxygenase in plant growth regulation. Regul Plant Growth Dev 40: 90–100


Smith YA (1977) Phenethylamine and related compounds in plants. Phytochem 16: 9–18


Yokoyama M (2005) Roles of 9,10-α-ketol linolenic acid (KODA), one of the oxylipins formed with 9-lipoxygenase in plant growth regulation. Regul Plant Growth Dev 40: 90–100

