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Isolation and stomatal opening activity of two oxylipins from *Ipomoea tricolor*

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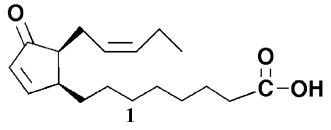
Abstract—Bioassay-guided fractionation of a 50% MeOH extract of *Ipomoea tricolor* enabled the isolation of two oxylipins, *cis*-12-oxophytodienoic acid (OPDA, **1**) and a novel monogalactosylmonoacylglyceride (**2**) containing OPDA, that acted as inducers of stomatal opening. These oxylipins enhanced stomatal opening of *Commelina communis* in darkness at micromolar concentrations.

Opening and closing of stomata are controlled by various conditions. The substance controlling stomatal closing has been elucidated to be abscisic acid (ABA).[1] Blue light strongly stimulates stomatal opening,[2] however, the substance controlling stomatal opening is not clear. We searched for endogenous substances that trigger stomatal opening from several plant extracts. The active fractions were followed using bioassay-guided purification to identify the active compounds.

In this study, the biological activity of a fraction was monitored by stomatal opening in the epidermis of *Commelina communis* in darkness.[3] An extract of *Ipomoea tricolor* strongly elicited stomatal opening in darkness. The aerial parts of 2-week-old *I. tricolor* (1930 g) were homogenized with 50% methanol. The homogenate was filtered, and the filtrate was dried. The residue was dissolved in 5% (v/v) acetonitrile to obtain a crude extract. The crude extract was applied to a Sep-Pak C-18 column and eluted with 5%, 30%, 60% and 100% acetonitrile. The active compounds were eluted with 60% acetonitrile and purified by two steps of RP-HPLC (Develosil ODS-HG-5) to give a known cyclopentenone fatty acid, *cis*-12-oxophytodienoic acid (**1**, 1.3 mg, 0.00007%) together with a new oxylipin (**2**, 1.8 mg, 0.00009%) as a colourless amorphous solid.



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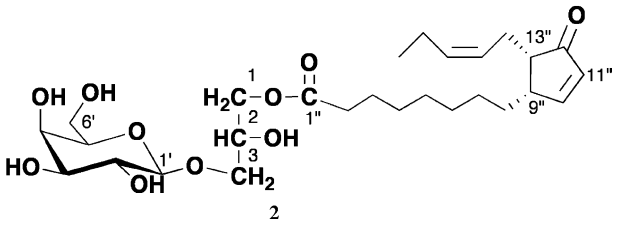

2

The structure of compound **1** was identified as an OPDA based on NMR and MS analysis. The ion peak of $[M+H]^+$ of **1** was detected by high-resolution positive ESI-TOF MS (*m*/*z* 293.2117 for $[M+H]^+$, Δ 0.6 mmu). The ^1H NMR analysis indicated that **1** is identical to OPDA as reported in the literature.[4]

The structure of compound **2** was determined by NMR (Table 1) and MS analysis, and by comparison with compound **1**. The molecular formula, $C_{27}H_{44}O_{10}$, of **2** was established by high-resolution positive ESI-TOF MS (*m*/*z* 551.2822 for $[M+Na]^+$, Δ −0.5 mmu).

The ^1H NMR spectrum of compound **2** indicated that **2** possessed an OPDA moiety. The ^1H–^1H COSY connectivities of C-1 to C-3 and C-1′ to C-6′ indicated the presence of a glycerol and a sugar component, respectively. The sugar was deduced to be β-galactopyranose based

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Table 1. ^{1}H and ^{13}C NMR data of **2**[a]

Position	^{1}H (J in Hz)	^{13}C	NOE correlations from ROESY
1α	4.15 dd(11.4, 4.6)	66.6	
1β	4.12 dd(11.4, 6.3)		
2	4.00 m	69.7	
3α	3.90 dd(10.4, 5.2)	71.9	H-1′
3β	3.65 dd(10.6, 4.7)		H-1′
1′	4.22 d (7.7)	105.4	H-3α, H-3β, H-3′, H-5′
2′	3.53 m	72.6	
3′	3.46 dd(9.6, 3.3)	74.9	H-1′, H-4′
4′	3.81 d (3.3)	70.3	H-3′, H-5′
5′	3.53 m	76.8	H-1′, H-4′
6′α	3.75 dd(11.3, 7.0)	62.5	
6′β	3.70 dd(11.3, 5.3)		
1″		175.5	
2″	2.35 t	34.9	
3″	1.61 m	25.9	
4″	1.34 m	30.2	
5″	1.34 m	28.6	
6″	1.34 m	30.7	
7″	1.34 m	29.0	
8″α	1.18 m	31.8	H-14″α
8″β	1.76 m		H-9″, H-14″α
9″	3.04 m	45.8	H-8″β, H-13″
10″	7.91 dd(5.8, 2.7)	170.3	
11″	6.15 dd(5.8, 1.7)	132.8	
12″	N.d.[b]		
13″	2.42 m	51.0	H-9″
14″α	2.17 m	25.0	H-8″α, H-8″β
14″β	2.49 m		
15″	5.41 m	133.8	
16″	5.41 m	134.9	
17″	2.07 m	21.7	
18″	0.97 t (7.6)	14.6	

[a] Recorded at 600 and 150 MHz for ^{1}H and ^{13}C NMR in CD_3OD, respectively.
[b] Not detectable.

on the ^{1}H–^{1}H coupling constants and NOE correlations from ROESY spectra in the sugar moiety. The HMBC correlation of H-1α (δ_H 4.15) and H-1β (δ_H 4.12) to C-1″ (δ_C 175.5) and H-1′ (δ_H 4.22) to C-3 (δ_C 71.9) and NOE correlations of H-1′ to H-3 revealed that the OPDA moiety was connected to C-1 and the sugar moiety to C-3. Therefore, compound **2** was assigned to be *sn*-1-*O*-(*cis*-12-oxophytodienoyl)-*sn*-3-*O*-(β-galactopyranosyl)glyceride.

The dose-dependent stomatal opening activities of OPDA and **2** are summarized in Figure 1.[3] OPDA (0.05 μg/mL, 0.2 μM, $P < 0.05$) and compound **2** (0.1 μg/mL, 0.2 μM, $P < 0.01$) induced significant stomatal opening in darkness. The response reached saturation at 0.1 μg/mL (0.4 μM) OPDA and 1 μg/mL (2 μM) compound **2**. Higher concentrations of OPDA (over 5 μg/mL, 20 μM) and **2** (10 μg/mL, 20 μM) did not enhance stomatal opening.

OPDA is a known precursor of jasmonic acid (JA), a wound and pathogen defense signal.[5] Compound **2** is an oxylipin containing OPDA that is structurally similar to three oxylipins isolated from *Arabidopsis thaliana*.[6,7]

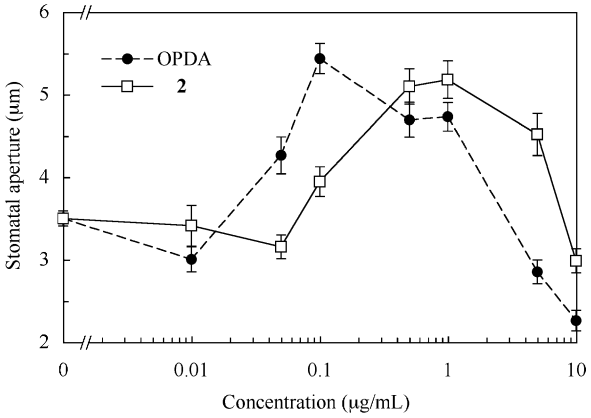

Figure 1. Effects of OPDA (closed circles) and compound **2** (open squares) on stomatal aperture in epidermis of *C. communis*.

The roles of these oxylipins, *sn*-1-*O*-(12-oxophytodienoyl)-*sn*-2-*O*-(hexadecatrienoyl)monogalactosyldiglyceride, *sn*-1-*O*-(12-oxophytodienoyl)-*sn*-2-*O*-(dinor-oxophytodienoyl)monogalactosyldiglyceride and *sn*-1, *sn*-2-di-*O*-(12-oxophytodienoyl)monogalactosyldiglyceride, are not clear. Considering that OPDA and **2** induce stomatal opening at similar concentrations, the activity of **2** is presumed to be due to OPDA released from **2** by enzymatic hydrolysis. A prospective function of **2** could thus be as a pool of OPDA. Although indole-3-acetic acid has been reported to induce stomatal opening,[8,9] the concentration (1 mM) is supra-physiological. On the other hand, OPDA showed stomatal opening activity at a significantly lower concentration (0.2 μM). These results suggest that OPDA plays a crucial role in stomatal opening, though it is not clear whether OPDA acts through a blue-light-induced stomatal opening pathway. The mechanism of OPDA action is under investigation. In addition, it is interesting that methyl jasmonate, a metabolite of JA, inhibits stomatal opening.[10] Further studies will be necessary to extend our knowledge of the relationship between OPDA and other jasmonates or phytohormones, such as ABA.

Acknowledgements

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4. The measurement of stomatal aperture was done as follows: The youngest fully expanded leaves from 3- to 4-week-old plants of *C. communis* were used. The experiments were initiated with closed stomata from leaves kept

in darkness. The abaxial epidermis was peeled and submerged in solutions containing 50 mM KCl, 10 mM MES–KOH (pH 6.2) and other test compounds. Peels were kept in darkness for 3 h. Following incubation, stomata were observed using a microscope fitted with a CCD camera. Images were captured and stomatal apertures were measured using Adobe Photoshop 7.0 software. Data presented are the means of 30 stomatal apertures with standard errors.

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