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YT performed the isolation and purification experiments; HS and JU elucidated chemical structures of active compounds; KM together with JU provided the idea, supervised and performed the research work, and wrote manuscript with YT

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ORIGINAL RESEARCH PAPER

Isolation and identification of polar auxin transport inhibitors from *Saussurea costus* and *Atractylodes japonica*

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Abstract
An intensive survey of naturally-occurring regulators of polar auxin transport (PAT) was conducted in two oriental medicinal species from the Asteraceae, *Saussurea costus* and *Atractylodes japonica*, using the radish hypocotyl bioassay system and physicochemical analyses. Costunolide and santamarine were identified as well as dehydrocostus lactone from *S. costus* roots, and atractylenolide II and (+)-eudesma-4(14),7(11)-dien-8-one from *Atractylodes japonica* rhizomes as physiologically novel compounds possessing inhibitory activities of PAT. Costunolide and santamarine showed ca. 40% inhibition of PAT in the radish hypocotyl segments at a dose of 2.5 µg/plant and 1 µg/plant, respectively. Inhibitory effects of atractylenolide II and (+)-eudesma-4(14),7(11)-dien-8-one were ca. 10 times lower than those of costunolide and santamarine. Structure–activity relationships and possible mechanisms to inhibit PAT are also discussed.

Keywords
atractylenolide II; *Atractylodes japonica*; polar auxin transport inhibitor; costunolide; eudesma-4(14),7(11)-dien-8-one; santamarine; *Saussurea costus*

Introduction

Auxin plays a crucial role in controlling various physiological phenomena in plant growth and development such as cell elongation, apical dominance, tropisms, floral bud formation, and vascular patterning. Auxin mediates different cellular responses based on its graded distribution between cells, local auxin biosynthesis, and directional cell-to-cell transport via carrier-mediated transport, being required for the establishment and maintenance of these auxin gradients in the plant axis. This unique transport system, called polar auxin transport (PAT), is considered to depend mainly on PIN-FORMED (PIN) auxin efflux carriers having a polar, plasma membrane localization [1–8]. It has also been demonstrated that PIN-dependent auxin efflux and local auxin response result in the apical–basal formation of the embryo and thus determine the axiality of the adult plant [9].

Chemicals functioning as regulators of PAT and/or anti-auxin are valuable for studying the mechanism of auxin action. The synthetic compounds of 2,3,5-triiodobenzoic acid (TIBA), *N*-(1-naphthyl)phthalamic acid (NPA), 9-hydroxyfluorene-9-carboxylic acid (HFCA), and methyl 2-chloro-9-hydroxyfluorene-9-carboxylate (morphactin) as

PAT inhibitors and *p*-chlorophenoxyisobutyric acid as anti-auxin have been well used [6]. By contrast, almost no naturally-occurring compounds exerting such physiological activities have ever been found. Only flavonoid compounds were found to act as negative regulators of auxin transport in vivo in *Arabidopsis* [10], whereas their function as modulators or regulators remains unconfirmed [11].

We have attempted to discover naturally-occurring regulators of PAT using an appropriate bioassay system with radish (*Raphanus sativus* L.) hypocotyl segments in several species of oriental medicinal Asteraceae, which contain various kinds of pharmacologically-active compounds not only for animals but also for plants. As a result, we have identified several physiologically-active compounds, such as a novel compound 3-hydroxy-4,6,7(H)-germacra-1(10),11(13)-dien-6,12-olide named artabolide [12], in addition to dehydrocostus lactone [decahydro-3,6,9-trismethylene-azulenol(4,5-b)furan-2(3H)-one, DHCL] and 4-hydroxy-β-thujone [4-hydroxy-4-methyl-1-(1-methylethyl)-bicyclo[3.1.0]hexan-3-one] [12,13]. The discovery of these active compounds led us to continue with the present study. Here, we report on the identification of costunolide and santamarine together with DHCL and of atractylenolide II and eudesma-4(14),7(11)-dien-8-one as PAT inhibitors, from *Saussurea costus* (Falc.) Lipsch. roots and *Atractylodes japonica* Koizumi rhizomes, respectively. Possible modes of action of these compounds are also discussed in relation to their chemical structures.

Material and methods

Plant material, extraction, and solvent fractionation

Dry *Saussurea costus* roots and *Atractylodes japonica* rhizomes were kindly provided by Koshiro Co., Ltd., Japan. Plant material (ca. 200 g) was homogenized with a blender and extracted three times with 2 L of 70% aqueous acetone. The filtered extract was concentrated in vacuo, giving an aqueous solution. Fractionation based on solvent partitioning against the aqueous solution was performed in the usual manner with ethyl acetate (EtOAc), providing EtOAc-soluble neutral (NE) and acidic (AE) fractions [14]. Because our preliminary experiments showed that active factors were found in the NE fraction instead of the AE fraction by the bioassay for PAT mentioned below, this NE fraction was dried over anhydrous Na_2SO_4 and then evaporated in vacuo to yield the active crude materials.

Purification of the PAT inhibitors from *Saussurea costus* using silicic acid adsorption column chromatography and thin-layer chromatography

The NE fraction from *S. costus* dissolved in a small amount of *n*-hexane was charged onto a column packed with silica gel (Wako-gel C 100; ϕ 3 × 90 cm) and eluted with *n*-hexane–EtOAc (100:0 → 90:10 → 80:20 → 70:30 → 60:40 → 50:50 → 40:60 → 20:80 → 0:100) in a stepwise gradient. Eluate with *n*-hexane–EtOAc 90:10 (v/v) and 50:50 (v/v) showed inhibitory activities of PAT on the bioassay system with the radish hypocotyl segments. The partially purified materials eluted with the *n*-hexane–EtOAc 90:10 (v/v) and 50:50 (v/v) were dissolved in a small amount of benzene, then charged onto a column packed with silica gel (Wako-gel C 100; ϕ 1 × 20 cm) and eluted with benzene–EtOAc (100:0 → 95:5 → 90:10 → 85:15 → 80:20 → 70:30 → 50:50 → 30:70 → 0:100) by increasing the EtOAc content of the mixture. Eluate with benzene–EtOAc 95:5 (v/v), from the partially purified materials eluted with the *n*-hexane–EtOAc 90:10, and that with benzene–EtOAc 70:30 (v/v), from the partially purified materials eluted with *n*-hexane–EtOAc 50:50, both contained active compounds.

For further purification, a silica gel GF_{254} (0.25-mm thickness; Merck Co., Ltd., Germany) was used for thin-layer chromatography (TLC). The partially purified active materials found in the fraction of benzene–EtOAc 95:5 (v/v) by silica gel column chromatography from the active fraction of *n*-hexane–EtOAC 90:10 (v/v) were purified on a preparative TLC developed with benzene–*n*-hexane–EtOAc (1:1:0.2, v/v/v). The active zone of R_F 0.39~0.66 was further purified with TLC developed with

n-hexane–chloroform–EtOAc (4:1:0.2, v/v/v). The active zones of R_F 0.47~0.55 and R_F 0.55~0.88 were both scraped off and eluted with EtOAc, giving the active materials upon evaporation.

The partially purified active material in the fraction of benzene–EtOAc 70:30 (v/v) on silica gel column chromatography from the active fraction of *n*-hexane–EtOAC 50:50 (v/v) was purified on TLC developed with *n*-hexane–chloroform–methanol (25:10:1, v/v/v). The active zone of R_F 0.14~0.29 was scraped off and eluted with EtOAc, giving an active material on evaporation.

Purification of the PAT inhibitors from *Atractylodes japonica* using silicic acid adsorption column chromatography and TLC

The NE fraction from *A. japonica* dissolved in a small amount of *n*-hexane was subjected to the same silica gel column chromatography (Wako-gel C 100; ϕ 3 × 90 cm) with the *n*-hexane–EtOAc solvent system as described above. Eluate with *n*-hexane–EtOAc 90:10 (v/v) was shown to contain active components. The partially purified components were further purified by silica gel column chromatography with the benzene–EtOAc mixture, as described above. Eluate with benzene–EtOAc 95:5 (v/v) and 90:10 (v/v) contained the active factors on the bioassay system with the radish hypocotyls.

The active material eluted with benzene–EtOAc 90:10 (v/v) using silica gel column chromatography was purified by TLC developed with *n*-hexane–EtOAc (95:5, v/v, multiple development). The active zone of R_F 0.17~0.25 was scraped off and eluted with EtOAc, giving an active material on evaporation. The active fraction obtained from the partially purified fraction of benzene–EtOAc 95:5 (v/v) was further purified on TLC developed with *n*-hexane–EtOAc (40:2, v/v, multiple development). The active zone of R_F 0.23~0.67 was further purified on a TLC developed with benzene–EtOAc (40:2, v/v, multiple development). The active zone of R_F 0.44~0.50 was scraped off and eluted with EtOAc, giving an active material on evaporation.

Spectral identification of compounds with inhibitory activity of PAT

The ^{1}H NMR spectra of the isolated compounds were recorded on a Bruker AVANCE-500 spectrometer (500 MHz). Deuterium-labelled chloroform ($CDCl_3$) and tetramethylsilan (TMS) were used as a solvent and as an internal standard, respectively. The stereochemical structures of these compounds were not determined in this study.

To elucidate the molecular mass of these compounds, GC-MS was performed on a Finnigan GCQ gas–liquid chromatography–mass spectrometry equipped with DB-5MS glass capillary column (0.251 mm × 30 m; J & W Scientific, USA), according to the method reported previously [13].

Bioassay for PAT

Following the method reported previously [8,13] with minor modifications, the system consisting of an ^{14}C-labeled IAA solution in the presence or absence of the test compounds in the bottom of 1.5-mL Eppendorf plastic tubes was introduced for the detection of the bioassay-derived PAT regulators. The plant extracts were placed into the bottom of the Eppendorf plastic tubes, dried in vacuo, and then dissolved with 25 µL of 1000-fold diluted ^{14}C-labeled IAA [(1-^{14}C)IAA with a specific activity of 55 mCi/mmol, 0.1 mCi/mL; American Radiolabeled Chemicals, Inc., USA]. Radish hypocotyl segments (each 20 mm in length), excised from 5 mm below the apical side of 6-day-old light-grown seedlings, were placed in the Eppendorf plastic tubes in down orientation of the apical side. Almost no auxin transport was observed when IAA was applied at the basal side of the segments in the same manner. Therefore, the ^{14}C-labeled IAA applied to the apical side was transported to the physiological apical–basal direction in this assay system as well as found in our previous results from a Fujix image analyzer of BAS 2000 (Fuji Photo Film Co., Ltd., Japan) [8] and from a liquid scintillation counter [13]. After incubation at 23.5°C for 18 h, a 2-mm piece of the opposite side of the segment

was cut; it was placed directly into a vial containing the liquid scintillation cocktails, and then its radioactivity was determined using a liquid scintillation counter.

Results

Identification of the polar auxin transport inhibitors from *Saussurea costus* and *Atractylodes japonica*

According to purification steps described in the "Material and methods", the active factors ultimately obtained from *S. costus* ran as a homogenous spot on analytical TLC, and they showed a single peak in GC-MS with the DB-5MS glass capillary column. The structure of the active factors found in R_F 0.47~0.55 and R_F 0.55~0.80 in TLC from the fraction eluted with benzene–EtOAc (95:5, v/v) were identified as costunolide and DHCL, respectively (Fig. 1), by comparison with the spectral data in the literature [15]. The structure of the active factor found in R_F 0.14~0.29 in TLC from the fraction eluted with benzene–EtOAc (70:30, v/v) was identified as santamarine by comparison with spectral data in the literature [15]. DHCL was already isolated and identified in our previous study as a bioassay-driven PAT inhibitor in *S. costus* and *Articum lappa* [13].

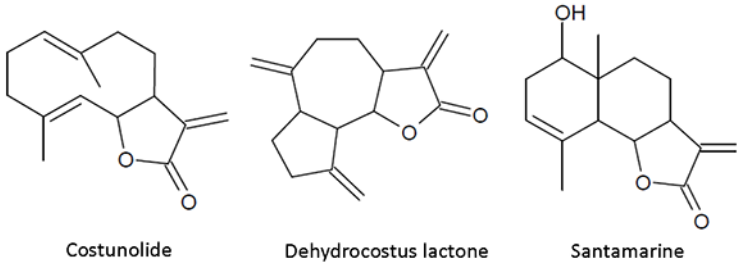

Fig. 1 Chemical structures of costunolide, dehydrocostus lactone, and santamarine identified as polar auxin transport inhibitors from roots of *Saussurea costus*. Stereochemical structures of these compounds have not been determined yet in this study.

After final purification by TLC, the active factor from *A. japonica* in the fraction of benzene–EtOAc 90:10 (v/v) from silica gel column chromatography was isolated as a homogenous spot in the zone of R_F 0.17~0.25 on TLC, with a single peak in GC-MS. This compound was identified as atractylenolide II (Fig. 2) by comparison with the spectral data reported in the literature [16]. From the fraction eluted with benzene–EtOAc 95:5 (v/v) on silica gel column chromatography, an active zone was finally detected in R_F 0.44~0.50 in the multiple-developed TLC with the solvent system of benzene–EtOAc (40:2, v/v) as a homogenous spot; it showed a single peak in GC-MS. This compound was identified as (+)-eudesma-4(14),7(11)-dien-8-one (Fig. 2) by comparison with spectral data in the literature [17].

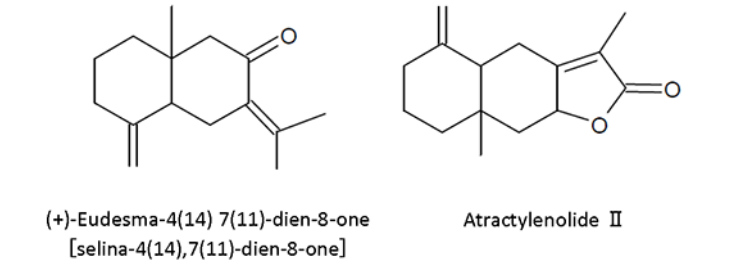

Fig. 2 Chemical structures of (+)-eudesma-4(14),7(11)-dien-8-one and atractylenolide II identified as polar auxin transport inhibitors from rhizomes of *Atractylodes japonica*. Stereochemical structures of these compounds have not been determined yet in this study.

Inhibitory effects of the isolated compounds on PAT in the radish hypocotyl segments

As shown in Fig. 3, both costunolide and DHCL were capable of significantly inhibiting PAT in radish hypocotyl segments. By applying these two compounds at 2.5 μg/plant, PAT was inhibited by ca. 40% when compared to the control. However, the inhibitory activity of santamarine was greater than that of costunolide and DHCL; the 1.5 μg/plant application of santamarine inhibited ca. 50% of PAT.

As shown in Fig. 4, the compound (+)-eudesma-4(14),7(11)-dien-8-one was effective at inhibiting PAT in the radish hypocotyl segments; it inhibited ca. 30% of PAT at 15 μg/plant. Similarly, atractylenolide II inhibited ca. 25% of PAT at 33.5 μg/plant in this bioassay system.

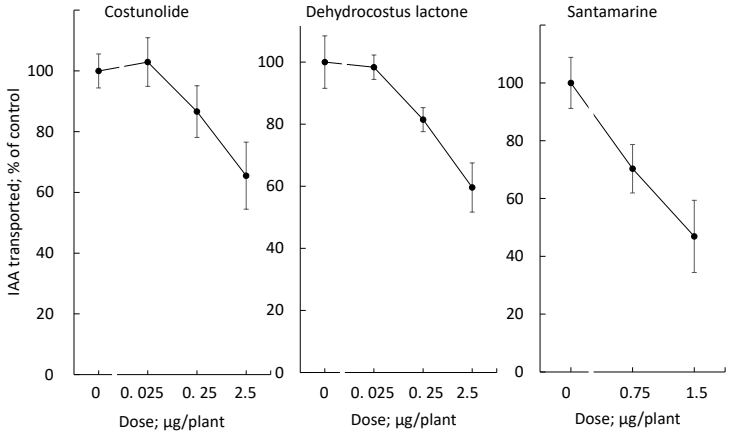

Fig. 3 Inhibitory effects of costunolide, dehydrocostus lactone, and santamarine from *Saussurea costus* on polar auxin transport of radish hypocotyl segments. Respective compounds were applied together with ^{14}C-labeled IAA to the apical side of hypocotyl segments. After 18-h incubation at 23.5°C, 2-mm piece of the opposite side of the segment was cut and directly put into a vial containing liquid scintillation cocktails, then radioactivity of the small slices was determined by a liquid scintillation counter. Values are expressed as % of control. Bars indicate the standard errors of the mean (n = 4).

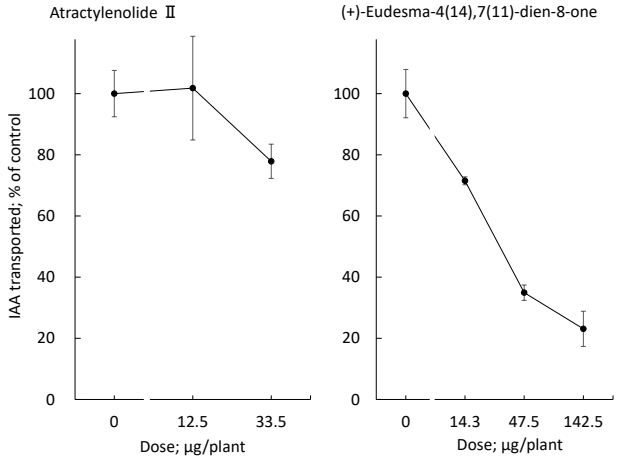

Fig. 4 Inhibitory effects of atractylenolide II and (+)-eudesma-4(14),7(11)-dien-8-one from *Atractylenodes japonica* on polar auxin transport of radish hypocotyl segments. Respective compounds were applied together with ^{14}C-labeled IAA to the apical side of hypocotyl segments. Others are the same as in Fig. 3.

According to the results shown in Fig. 3 and Fig. 4, the compounds found in this study are significant PAT inhibitors that are naturally occurring. However, the effect is not powerful as compared to the synthetic PAT inhibitors, NPA, TIBA, and HFCA, as reported previously [13].

Discussion

The utility of the radish hypocotyl bioassay for determining naturally-occurring PAT inhibitors as well as synthetic ones has been demonstrated in other works [13]. Using this bioassay system, DHCL and a novel germacranolide-type of sesquiterpene lactone named artabolide and 4-hydroxy-β-thujone have been isolated and identified from the two oriental medicinal Asteraceae plants, *S. costus* and *Artemisia absinthium*, respectively [12,13]. In the present study, costunolide and santamarine together with DHCL were successfully identified as PAT inhibitors from *S. costus*. Although costunolide and santamarine have already been isolated from *Costus lappa* [18,19] and *Chrysanthemum parthenium* [20], respectively, this is the first report to describe that these sesquiterpene lactones are PAT inhibitors from *S. costus*.

In our previous studies, it has been suggested an important role of the α-methylene-γ-lactone moiety in artabolide is for inhibiting PAT, because hydroxypelenolide, ketopelenolide a, and ketopelenolide b, which have α-methyl-γ-lactone moiety instead of the α-methylene-γ-lactone one, have a much lesser inhibition activity [12]. All of the naturally-occurring PAT inhibitors, namely costunolide, santamarine, and DHCL, isolated from *S. costus* showed the characteristic structure of a sesquiterpene lactone with the α-methylene-γ-lactone moiety, thereby strongly supporting our previous suggestion [12,13]. The structural importance of the γ-lactone moiety of DHCL for its biological activity was also suggested for the seed germination of parasitic plant *Orobanche cumana* [21]. Costunolide as well as DHCL have been reported to be part of a major functional group conferring various biological activities in the sesquiterpene lactones in both animals and plants [22–27]. Hence, it may be possible to explain why the exocyclic methylene double bond conjugated with a γ-lactone ring is essential for the biological activities of sesquiterpene lactones.

The compounds (+)-eudesma-4(14),7(11)-dien-8-one [selina-4(14),7(11)-dien-8-one], and atractylenolide II were isolated as bioassay-driven PAT inhibitors from the rhizomes of *A. japonica*. Eudesma-4(14),7(11)-dien-8-one was first isolated and identified as a naturally-occurring sesquiterpenoid from the rhizomes of *Asarum caulescens* (Aristolochiaceae) [28], and later found in the hexane extract from *A. japonica* [29]. Furthermore, it has been shown to have significant anti-inflammatory activity [17], whereas atractylenolide II is a sesquiterpene compound with anti-proliferative activity isolated from the rhizome of *Atractylodes macrocephala* ("Baizhu" in Chinese) [30]. Although the inhibitory activity of PAT of both these two sesquiterpene lactones is

lower than those with an α-methylene-γ-lactone moiety mentioned above, this is the first report to reveal these are PAT inhibitors in *A. japonica*. The chemical structure of atractylenolide II also supports the proposal for an important role of the α-methylene-γ-lactone moiety in the biological activity controlling PAT in plants. According to our preliminary studies (data not shown), costunolide, DHCL, and santamarine from *S. costus* as well as (+)-eudesma-4(14),7(11)-dien-8-one [selina-4(14),7(11)-dien-8-one] and atractylenolide II from *A. japonica* are difficult to dissolve in water. Among them, santamarine has a hydroxyl group in the C-1 position, making it more effective than the others. Further studies on sesquiterpene lactones with an α-methylene-γ-lactone moiety on penetration of, or uptake by, plant tissues in relation to their biological activities are required.

Although PAT-based hormonal signaling in plants still remains unclear, recent molecular studies indicate an important role for PIN proteins as efflux carriers located on the plasma membrane in controlling the direction of cell-to-cell auxin transport in realizing the developmental processes, such as embryogenesis, morphogenesis, organogenesis, and tropisms [3]. It has been demonstrated that the PIN-dependent auxin efflux and the local auxin response lead to the apical–basal formation of the embryo and thus determine the axiality of the adult plant [9]. In *Arabidopsis*, the importance of PAT for controlling its leaf vascular patterning is also suggested, since subcellular PIN polarity indicates that auxin is directed to a distinct convergence point in the epidermis, from where it defines the positions of major veins [2]. The application of flavonoids promotes asymmetric PIN shifts during gravity stimulation, indicating that a redirected basipetal auxin stream is necessary for root bending [31,32]. The mode of action of sesquiterpene lactones with the α-methylene-γ-lactone moiety shown in this study to inhibit PAT remains unclear. DHCL has been demonstrated to suppress gene expression of *PsPIN1* and *PsAUX1* encoding the auxin influx carrier in etiolated pea seedlings (Toda et al., personal communication, 2017). Therefore, studies of sesquiterpene lactones with an α-methylene-γ-lactone moiety and their relevance to the polarity maintenance of auxin carrier proteins, such as PIN and AUX1 proteins, using antibodies will be required in the near future.

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Izolacja i identyfikacja inhibitorów polarnego transport auksyny z *Saussurea costus* i *Atractylodes japonica*

Streszczenie

Przeprowadzono przegląd naturalnie występujących regulatorów polarnego transportu auksyny w roślinach mających zastosowanie w medycynie orientalnej, *Saussurea costus* i *Atractylodes japonica*, stosując powszechnie uznany biotest hypokotyla rzodkiewki (*Raphanus sativus* L.) i analizy fizykochemiczne. Zidentyfikowano kostunolid i santamarynę jak również lakton dehydrokostusowy i atraktylenolid II i (+)-eudesma-4(14),7(11)-dien-8-on jako nowe związki mające fizjologiczne właściwości inhibitorów polarnego transportu auksyny, kolejno z korzeni *Saussurea costus* i kłączy *Atractylodes japonica*. Kostunolid i santamaryna wykazały w około 40% hamowanie polarnego transportu auksyny w segmentach hypokotyla rzodkiewki przy stężeniu 2.5 µg/roślinę i 1 µg/roślinę. Hamujące działanie atraktylenolidu II i (+)-eudesma-4(14),7(11)--dien-8-onu było około 10 razy mniejsze niż kostunolidu i santamaryny w badanym procesie. W pracy przedyskutowano zależności między aktywnością a strukturą chemiczną w/w związków i możliwy mechanizm ich hamującego działania na polarny transport auksyny.