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Heliolactone, a non-sesquiterpene lactone germination stimulant for root parasitic weeds from sunflower

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Abstract

Root exudates of sunflower (*Helianthus annuus* L.) line 2607A induced germination of seeds of root parasitic weeds *Striga hermonthica*, *Orobancha cumana*, *O. minor*, *O. crenata*, and *Phelipanche aegyptiaca*. Bioassay-guided purification led to the isolation of a germination stimulant designated as heliolactone. FT-MS analysis indicated a molecular formula of $C_{20}H_{24}O_6$. Detailed NMR spectroscopic studies established a methylfuranone group, a common structural component of strigolactones connected to a methyl ester of a C_{14} carboxylic acid via an enol ether bridge. The cyclohexenone ring is identical to that of 3-oxo- α -ionol and the other part of the molecule corresponds to an oxidized carlactone at C-19. It is a carlactone-type molecule and functions as a germination stimulant for seeds of root parasitic weeds. Heliolactone induced seed germination of the above mentioned root parasitic weeds, while dehydrocostus lactone and costunolide, sesquiterpene lactones isolated from sunflower root exudates, were effective only on *O. cumana* and *O. minor*. Heliolactone production in aquacultures increased when sunflower seedlings were grown hydroponically in tap water and decreased on supplementation of the culture with either phosphorus or nitrogen. Costunolide, on the other hand, was detected at a higher concentration in well-nourished medium as opposed to nutrient-deficient media, thus suggesting a contrasting contribution of heliolactone and the sesquiterpene lactone to the germination of *O. cumana* under different soil fertility levels.

Keywords: Sunflower; *Helianthus annuus*; Asteraceae; *Orobancha cumana*, Orobanchaceae;; Strigolactone; Carlactone

1. Introduction

Root parasitic weeds of the genera *Striga*, *Orobanche*, and *Phelipanche* adversely affect many important food crops. The germination of parasitic root weeds depends on chemical signals released from host and non-host plants into the rhizosphere. Since the identification of strigol (Cook et al., 1972), a series of analogous compounds have been isolated. Strigol and analogous compounds, generally known as strigolactones (SLs) (Bulter, 1995), share a common structure composed of a tricyclic lactone (the ABC part) connected via an enol ether bridge to a methylfuranone group (the D-ring). SLs were recently identified as a hyphal branching factor for arbuscular mycorrhizal (AM) fungi and a new class of plant hormones that play a key role in the regulation of plant architecture (Ruyter-Spira et al., 2013). Recently, it was demonstrated that SLs were biosynthesized from carlactone (**1**) (Seto et al., 2014). The latter has a SL-like carbon skeleton and is synthesized from β -carotene catalyzed by sequential reactions of three biosynthetic enzymes: D27, carotenoid cleavage dioxygenase 7 (CCD7), and CCD8 (Alder et al., 2012).

The root holoparasitic plant *O. cumana* presents a serious constraint to sunflower (*Helianthus annuus* L.) production in Southeast Europe, the Middle East, and Southwest Asia (Parker, 2013). The sesquiterpene lactones (STLs), dehydrocostus lactone (DCL, **2**) (Joel et al., 2011), costunolide (**3**), tomentosin, and 8-epixanthatin (Raupp and Spring, 2013), potent germination stimulants for *O. cumana* seeds instead of SLs, were isolated from sunflower root exudates. Yoneyama et al. (2011) reported the detection of LC-MS/MS signals corresponding to the SLs 5-deoxystrigol (5-DS, **4**) and aleictrol (**5**) in sunflower root exudates, but the eluted fractions did not induce *O. minor* seed germination. Considering the biological importance of SLs and their ubiquitous distribution in the plant kingdom (Ruyter-Spira et al., 2013), the production of SLs or its related molecule(s) by sunflowers is logical. In a preliminary experiment and as described below, it was observed that sunflower root exudates induced the germination of *S. hermonthica* seeds, whereas the commercially available STLs **2** and **3** did not. Failure of these STLs to induce germination of *S. hermonthica* led to probing sunflower root exudates for non-STLs with germination inducing activity. This paper reports the isolation and identification of a carlactone-like molecule named heliolactone (**6**) from sunflower root exudates, its germination inducing activity on the seeds of several root parasitic weeds, and the effects of phosphorus and nitrogen on its production.

2. Results and discussion

2.1. Isolation and structural determination of heliolactone (**6**)

Bioassay-guided purification of sunflower root exudates by silica gel column chromatography led to the separation of active fractions that induced germination of *S. hermonthica* seeds. Fraction 10 (40% EtOAc in hexane), the most active fraction, contained a novel germination stimulant with a molecular mass of 360 and a major fragment ion at m/z 97, characteristic of SLs, in LC-MS/MS analysis. The stimulant was purified with reversed-phase HPLC, followed by chiral HPLC to give a nearly pure compound. The FT-MS analysis of the stimulant established the sodium adduct ion at m/z 383.1457 $[M+Na]^+$ calculated for $C_{20}H_{24}O_6Na$. The 1H NMR spectrum of the stimulant in $CDCl_3$ exhibited fourteen signals (Table 1). The presence of a methyl resonance at δ_H 2.04 and two methine signals at δ_H 6.15 and 6.95, typical of the D-ring in SLs such as alectrol (**5**) (Table 1) (Ueno et al., 2011; Nomura et al., 2013), indicated that the stimulant was analogous to SLs as predicted by a major fragment ion at m/z 97 in its mass spectrum, as well as germination inducing activity toward *S. hermonthica* seeds. The chemical shift of an oxygen-bearing olefinic methine resonance (δ_H 7.53), corresponding to the enol ether moiety adjacent to the carbonyl group in SLs, also supported the similarity of the stimulant to SLs. However, the signal appeared as a singlet in the stimulant, although the H-6' resonance appeared as a doublet by coupling with H-3a in SLs. In addition, two olefinic methine signals (δ_H 6.24 and 6.25) and a methoxycarbonyl resonance (δ_H 3.75) were observed. These data suggested that the stimulant had a carlactone-like carbon skeleton in which a methyl group at C-19 was oxidized and methylated to a methoxycarbonyl group. The remaining three methyl signals (δ_H 0.96, 1.04, and 1.91), two non-equivalent methylene resonances (δ_H 2.09 and 2.34), and two methine signals (δ_H 2.57 and 5.91) were similar to those of the trimethylcyclohexenone ring in 9,10-dihydroxy-4,7-megastigmadien-3-one (**7**) (Greger et al., 2001) (Table 1) and 1'-deoxyabscisic acid (1'-deoxy-ABA, **8**) (Todoroki et al., 1995), thus suggesting the presence of a 3-oxo- ϵ -end group instead of a β -end group in carlactone (**1**) and β -carotene.

In order to separate two partially overlapping olefinic methine resonances (H-7 and H-8) occurring in $CDCl_3$, 1H and ^{13}C NMR spectra of the stimulant were measured in C_6D_6 (Table 2). The methine signals were successfully separated and detected at δ_H 6.46 and 6.40. The *E* configuration of the double bond (C-7 and C-8) was deduced from the coupling constants (both 15.9 Hz). A DIFNOE experiment also supported the partial structure; irradiation of the H-8 proton (δ_H 6.40) caused an NOE enhancement of the H-6

resonance (Fig. 2). The ^1H and ^{13}C NMR spectroscopic data corresponding to the six-membered ring of the stimulant were similar to those of isophorone (3,5,5-trimethylcyclohex-2-en-1-one, **9**) and acetylated 3-oxo- α -ionol β -D-glucoside (Pabst et al., 1992). Moreover, those corresponding to the enol ether bridge and the D-ring were very similar to those for 5-deoxystrigol (**4**), measured in the same solvent. The ^{13}C NMR signals corresponding to the diene moiety in the acyclic C_5 branched chain were comparable with those for carlactone measured in CD_2Cl_2 (Alder et al., 2012). The connections of the structural portions mentioned above were unambiguously confirmed by HMBC data (Fig. 2). The HMBC experiment confirmed that the H-10 resonance correlated with the C-8, C-9, C-11, and C-19 signals, the H-8 signal correlated with the C-6, C-7, C-10, and C-19 resonances, and the H-6 signal correlated with the C-7 and C-8 resonances. Attachment of the methoxy group to the carbonyl group was confirmed by the HMBC correlation of the methyl proton (19-OMe) signal to the C-19 signal. All ^1H and ^{13}C NMR resonances were assigned on the basis of the DEPT, DIFNOE, ^1H - ^1H COSY, HMQC and HMBC data as shown in Table 2. The structure of the novel sunflower stimulant was therefore established to be (2*E*,3*E*)-methyl 2-[(4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy]methylene}-4-(2,6,6-trimethyl-4-oxocyclohex-2-en-1-yl)but-3-enoate., and is assigned the name heliolactone (**6**).

In the DIFNOE spectra, the upfield methyl proton (δ_{H} 0.78, C-16) exhibited NOEs to the methylene protons at C-2, the methine protons at C-6, C-7 and C-8. On the other hand, the downfield methyl proton (δ_{H} 0.79, C-17) had NOEs to the upfield proton at C-2 (δ_{H} 2.05) and C-6. These results implied that the favoured ring conformation of **6** was the half-chair with the pseudo-axial C_5 branched diene moiety (Fig. 2), similar to the more favoured conformation of ABA (Todoroki and Hirai, 2000).

The circular dichroism (CD) spectrum of heliolactone (**6**) showed positive maxima at 219 nm and 327 nm and a negative maximum at 246 nm. Since heliolactone (**6**) has stereocenters at C-6 and C-11, the CD curve of **6** is regarded as the sum of the curves of the different chromophoric pairs, a cyclohexenone/dienoate moiety presented in a similar way to ABA, and a furanone/enone moiety in a similar way to SLs. The CD curve of (1'*S*)-(-)-1'-deoxy-ABA [(-)-**8**] had positive maxima at 216 nm and 316 nm, and a negative maximum at 264 nm (Todoroki et al., 1995). (6*S*)-3-Oxo- α -ionol (Pabst et al., 1992) and opuntiside B (**10**), isolated from *Opuntia ficus-indica* var. *saboten* (Saleem et al., 2006), also showed a positive maximum above 300 nm derived from the $n\text{-}\pi^*$ transition of the cyclohexenone (Koreeda et al., 1973); thus the (6*S*)-configuration in **6** was assigned. On the other hand, a negative Cotton effect

around 250 nm suggested the *R* configuration at C-2' in SL [at C-11 in carlactone (**1**) and heliolactone (**6**)] (Welzel et al., 1999). The maximum $\Delta\epsilon$ value for furanone (within ± 5) including C-11, however, was smaller than that for cyclohexenone including C-6 [for instance, -24.2 at 264 nm for (-)-**8**] (Gawronski et al., 1996; Frischmuth et al., 1993; Todoroki et al., 1995). In addition, (11*R*)-carlactone (**1**) showed a positive maximum ($\Delta\epsilon = +4$) at 270 nm (Seto et al., 2014). Accordingly, the absolute stereochemistry at C-11 was not determined via CD because the curve derived from the furanone may be hidden via overlap with the curve derived from the cyclohexenone/dienoate chromophoric pair.

2.2. Response of seeds of root parasitic weeds to heliolactone (**6**) and sesquiterpene lactones **2** and **3**

The germination inducing activities of heliolactone (**6**) on *S. hermonthica*, *O. cumana*, *O. minor*, *O. crenata*, and *P. aegyptiaca* seeds are shown in Fig. 3. Helioactone (**6**) induced the germination of all the parasitic weed species tested in this study and its activities at 1 μ M were as high as those of GR24, a synthetic SL, at the same concentration. STLs, DCL (**2**) and costunolide (**3**) induced the germination of *O. cumana* and *O. minor* seeds, but the effects on the other parasite seeds were negligible. The wider spectrum of heliolactone (**6**) is evidence that germination inducing activity of sunflower root exudates is, at least in part, due to heliolactone (**6**). The narrower spectrum of STLs may imply an adaptation of some *Orobanch*e species to germinate in the soil with host roots exuding limited SL. Neither heliolactone (**6**) nor STLs induced germination of *S. gesnerioides* seeds although the seeds responded to *ent*-2'-*epi*-orobanchol (data not shown), which is in line with strict structural requirements of SLs for germination induction of the seeds (Nomura et al., 2013).

2.3. Effects of phosphorus and nitrogen on the production of heliolactone (**6**) and costunolide (**3**)

As has been well documented, SLs function as a hyphal branching factor for AM fungi. Therefore, their biosynthesis is promoted in host plants under low phosphorus conditions. Nitrogen deficiency is also reported to affect the production of SLs (Yoneyama et al., 2012). In sunflower root exudates, a substantial amount of heliolactone (**6**) was observed when seedlings were grown in tap water (Fig. 4A). Supplementation of phosphorus (Fig. 4B) or nitrogen (Fig. 4C) drastically reduced the exudation of **6**. Removal of both phosphorus and nitrogen from half-strength Hoagland's solution also increased the production of **6** (data not shown). On the other hand, increase of costunolide (**3**) was detected while the

heliolactone (**6**) content was below the detection limit under conditions of high phosphorus and nitrogen content (Fig. 4D). DCL (**2**) content, albeit it was the first STL identified in sunflower root exudates as a germination stimulant for *O. cumana* (Joel et al., 2011), was negligible under any aquaculture conditions tested in the experiments. Moreover, none of the known SLs was detected even under phosphorus-and nitrogen-deficient conditions in LC-MS/MS analysis using the parent ion mode. These findings suggest the involvement of **6** in the rhizosphere communication between sunflower roots and neighbouring microorganisms including AM fungi. Inability of DCL (**2**) to induce hyphal branching in AM fungi (Akiyama et al., 2010) also supports the rhizospheric function of heliolactone (**6**).

2.4. Carotenoid precursors of heliolactone (**6**)

The production of heliolactone (**6**) was inhibited by a treatment with fluridone, a carotenoid biosynthesis inhibitor (data not shown), indicating that heliolactone (**6**) is derived from carotenoid as well as SLs (Matusova et al., 2005). Carlactone (**1**), a novel apocarotenoid with a comparatively simple structure and SL-like biological activities, was produced *in vitro* from β -carotene and its formation is catalyzed by the three enzymes, D27, CCD7, and CCD8 (Alder et al., 2012). Recently, carlactone (**1**) was identified in the roots of rice and *Arabidopsis thaliana*, and its conversion to *ent*-2'-*epi*-5-DS (*ent*-2'-*epi*-**4**) was demonstrated in a rice *d10* mutant by tracer experiments (Seto et al., 2014). The conversion, despite lack of information on enzymes including CYP711A/MAX1 catalysing the reactions, likely proceeds through coordinated oxidation steps at C-18 and C-19 in **1**, followed by the formation of the B- and C-rings in a stereo-controlled manner. Heliolactone (**6**) is oxidized at C-19, whereas carlactone (**1**) is not, suggesting that the oxidation of C-19 into the carboxylate is involved in SL biosynthesis. However, modification of the six-membered rings between **1** and **6** are remarkably different. Specifically, the former contains a β -end group while the latter contains a 3-oxo- ϵ -end group as the C₉ end group of carotenoids. The difference poses some possibilities of the carotenoid precursor of heliolactone (**6**). If carlactone (**1**) generated from β -carotene is a precursor of **6**, oxygen incorporation at C-3 and isomerization of the six-membered ring would be needed in addition to the oxidation at C-19. Since no SL was identified in the sunflower root exudates by bioassay-guided purification and LC-MS/MS, a mutation may take place in the enzyme(s) involved in SL biosynthesis and result in the change of oxidation sites. Alternatively, other oxidases and isomerases would act in heliolactone (**6**) biosynthesis in compensation for the lack of SL biosynthetic enzymes. Another

possibility is that carlactone (**1**) and β -carotene would not be precursors of heliolactone (**6**); that is, a carlactone-like compound can be also generated from other carotenoids, if D27, CCD7, and CCD8 or their respective homologue(s) act on these substrates in a similar mechanism to β -carotene. Oxidation and isomerization would be required for the production of heliolactone (**6**) when 3-hydroxy-carlactone is generated from zeaxanthin and lutein, which contain the 3-hydroxylated β -end group. Most recently, evidence to support the biosynthetic pathway was reported by Bruno et al. (2014), who demonstrated that CCD7s of Arabidopsis and tomato cleaved 9-*cis*-zeaxanthin to yield 9-*cis*-3-hydroxy- β -apo-10'-carotenal. Other possibilities include lutein with the 3-hydroxylated ϵ -end group as a precursor of an allyl-hydroxy carolactone-like compound, or that a carlactone isomer is generated from α -carotene and zeinoxanthin with an ϵ -end group and is subsequently oxidized at C-3. The enzymatic cleavage of 9-*cis*-lutein to 9-*cis*-3-hydroxy- ϵ -apo-10'-carotenal and 3-hydroxy- β -ionone was also reported (Bruno et al., 2014). In this case, however, the stereochemistry at C-6 would be discussed carefully because the CD spectrum of heliolactone (**6**) suggests the *S* configuration at C-6, whereas the stereochemistry at C-6 in the ϵ -end group of carotenoids is *R* (Isler, 1971). Moreover, the modification of the six-membered ring by oxidation and/or isomerization could hamper the formation of the BC rings of SL.

3. Concluding Remarks

In conclusion, a novel germination stimulant for parasitic weeds, heliolactone (**6**), was isolated from sunflower root exudates. The structural core of heliolactone (**6**) is like that of carlactone (**1**). Since none of SLs identified so far have the oxygen atom at C-6, the corresponding position at C-3 in heliolactone (**6**), the carotenoid precursor of **6** may be different from that of carlactone (**1**). Exudation of known STL costunolide (**3**) increased under well-nourished conditions while that of heliolactone (**6**) was promoted under nutrient-deficient conditions, suggesting involvement of **6** in the rhizosphere communication between sunflower roots and neighbouring microorganisms. The extended responsiveness to different types of chemicals, heliolactone (**6**) and STLs, whose production by sunflower is contrasting under different nutritional conditions, may be an adaptation of *O. cumana* to ensure germination and parasitization to sunflowers under a broad range of soil fertility conditions.

4. Experimental

4.1. Apparatus

NMR spectra were recorded in CDCl₃ on a JNM-AL 300 spectrometer at 300 MHz for ¹H, and in C₆D₆ on a JNM-ECA 600 spectrometer (JEOL, Tokyo, Japan) at 600 MHz for ¹H and at 150 MHz for ¹³C. All NMR chemical shifts were referenced to tetramethylsilane or C₆D₆ (δ_{H} 7.15, δ_{C} 128.0). UV and CD spectra were acquired with a V-630 spectrophotometer and a J-805 spectropolarimeter (JASCO, Tokyo, Japan), respectively. LC-MS/MS analyses were performed using an LC-MS/MS system (Waters, Milford, MA) consisting of an Acquity Ultra Performance liquid chromatography (UPLC) and an Acquity quadrupole tandem mass spectrometer (TQ Detector). Data acquisition and analyses were performed using MassLynx 4.1 software (Waters, Milford, MA). High-resolution FT-mass spectra were obtained using a LTQ Orbitrap Discovery (Thermo Fisher Scientific K.K., Yokohama, Japan).

4.2. Chemicals

DCL (**2**) and costunolide (**3**) were purchased from Wako Chemical Industry (Osaka, Japan) and Extrasynthese (Genay, France), respectively. Isophorone (**9**) and all organic solvents were obtained from Tokyo Chemical Industry (Tokyo, Japan) and Kanto Chemical (Tokyo, Japan), respectively. GR24 was kindly provided by Prof. Zwanenburg (Radboud University Nijmegen, the Netherlands). *Ent*-2'-*epi*-orobanchol was isolated from cowpea root exudates as in Ueno et al., 2011. A racemic mixture of 5-DS (**4**) was synthesized as reported previously (Nomura et al., 2013).

4.3. Isophorone (**9**)

¹H NMR (600 MHz, C₆D₆): δ 0.72 (6H, s, 5-Me₂), 1.42 (3H, dt, J = 1.4, 0.8 Hz, 3-Me), 1.56 (2H, br.s, H-4), 1.99 (2H, s, H-6), 5.83 (1H, tq, J = 1.4, 1.4 Hz, H-2).

¹³C NMR (150 MHz, C₆D₆): δ 23.9 (q, 3-Me), 28.1 (q, 5-Me₂), 33.1 (s, C-5), 44.8 (t, C-4), 50.8 (t, C-6), 125.9 (d, C-2), 158.0 (s, C-3), 197.5 (s, C-1).

4.4. Plant materials

Sunflower, line 2607A, seeds were obtained from Dobrudja Agricultural Institute, Bulgaria. Seeds of *O. cumana* and *O. minor* were collected from sunflowers in the Dobrich region of Bulgaria and red clovers in Yokohama, Japan, respectively. Seeds of other parasitic weeds *O. crenata*, *P. aegyptiaca*, *S. hermonthica*, and *S. gesnerioides* were supplied by Dr. Daniel Joel, Newe-Ya'ar Research Center, Israel, Dr. Yaakov Goldwasser, The Hebrew University of Jerusalem, Israel, Prof. Abdel Gabar Babiker, Sudan University of Science and Technology, Sudan, and Dr. Satoru Muranaka, International Institute of Tropical Agriculture, Nigeria, respectively.

4.5. Germination bioassay

The germination assay was conducted as reported by Sugimoto et al. (2003). The surface sterilized seeds were pretreated (conditioned) for 8–12 days at 30 °C for *S. hermonthica*, at 28 °C for *S. gesnerioides*, and at 23 °C for other parasites on 8 mm glass fibre filter paper disks (*ca.* 50 seeds each) placed on distilled water-saturated filter paper. Aliquots (20 µL) of diluted test solutions were assayed by applying them to the conditioned seeds on 8-mm disks. The treated seeds were incubated at the same temperature as that used for conditioning and were microscopically examined for germination (radicle protrusion) 24 to 72 h later.

4.6. Hydroponic culture and collection of root exudates of sunflower

Fifteen sunflower seedlings were grown hydroponically in a 30 L tank filled with modified 40% Long Ashton nutrient solution, where the phosphate and nitrogen concentrations were adjusted to 0.053 mM and 2 mM, respectively. The medium was continuously circulated with a pump, and sunflower root exudates of 2 to 8 week old seedlings were collected by adsorption on activated charcoal (12 g) as described previously (Ueno et al., 2011). The charcoal was collected every week and soaked in acetone (80 mL) for a couple of days at 4 °C. After evaporating the acetone, the residual aqueous solution (*ca.* 20 mL) was treated three times with EtOAc (total volume 20 mL), and the organic layer was washed with 0.2 M K₂HPO₄ (pH 8.3), dried over Na₂SO₄, and concentrated *in vacuo*.

4.7. Isolation of germination stimulants

The concentrated EtOAc extracts of the sunflower root exudates obtained from 28 replications were combined and subjected to silica gel column chromatography, CC (20 g) with a stepwise elution of

hexane/EtOAc (100:0–0:100, 10% step, 50 mL $\times$ 2 in each step) and subsequent MeOH to yield fractions 1–23. Distinct germination inducing activity toward *O. cumana* and *O. minor* was detected in fractions 9 and 10 (40% EtOAc) and 16 (70% EtOAc). The germination inducing activity toward *S. hermonthica* was the most active in fraction 10. Therefore, it was concentrated *in vacuo* and subjected to reversed-phase HPLC on a 250 $\times$ 20 mm i.d., 5 μ m, CAPCELL PAK C18 UG120 S5 (Shiseido, Yokohama, Japan) with MeOH in H₂O (60:40, v/v) at 5 mL/min with monitoring at 235 nm. The active ingredients toward *S. hermonthica* were eluted at 24 min and 43 min. The stimulant eluted at 43 min showed a pseudomolecular ion at m/z 361 and fragment ions at m/z 233 and 97 in daughter ion scan analysis of m/z 361 in LC-MS/MS analyses, as described above. The stimulant that eluted at 24 min showed a pseudomolecular ion at m/z 377. Conversion of the stimulant of m/z 361 to that of m/z 377 was observed in the purification process, indicating that the latter is an artefact, and is probably the oxidized product of the former. Accordingly, the purification was concentrated on the stimulant eluted at 43 min. The stimulant eluted at 43 min was subsequently subjected to chiral HPLC on a 250 $\times$ 10 mm i.d., 5 μ m, CHIRALPAK IC (Daicel, Osaka, Japan) with EtOH in hexane (50:50, v/v) at 1 mL/min with monitoring at 235 nm. Stimulant **6**, which eluted at 41 min was collected, concentrated, and passed through a silica gel column with EtOAc in CHCl₃ (25:75, v/v).

4.8. Heliolactone (**6**)

HRMS m/z calcd for C₂₀H₂₄O₆Na: 383.1471, found 383.1457.

For ¹H NMR (300 MHz, CDCl₃) ¹H and ¹³C NMR (600 and 150 MHz, C₆D₆) spectroscopic data, see Tables 1 and 2. UV(MeCN) λ_{max} 234 nm. CD (MeCN, c 0.000069) λ_{ext} ($\Delta\epsilon$) 327 (+ 0.96), 246 (-7.8), 219 (+8.3) nm.

4.9. Extraction of STLs and heliolactone (**6**)

In small containers filled with modified 40% Long Ashton nutrient solution with nitrogen concentration of 2 mM (1 L), four sunflower seedlings were grown with a pump for aeration for 13 days. The solution was replaced by tap water (-P, -N), tap water supplemented with 0.53 mM Na₂HPO₄ (+P, -N), tap water supplemented with 1 mM NH₄NO₃ (-P, +N) or modified 40% Long Ashton nutrient solution (+P, +N) and the plants were allowed to grow for 3 days, prior to culture filtrate collection. The culture filtrates were

subsequently subjected to liquid-liquid partitioning using EtOAc three times (total volume 500 mL). In an alternative method, sunflower seedlings were grown in falcon tubes with quarter-strength Hoagland's solution for a week and then transferred to glass test tubes containing 100 mL of half-strength Hoagland's solution, nitrogen-limited and/or phosphate-limited (1/10) media. Three days later, the culture media were replaced with 150 mL of full nutrient, nitrogen-free, phosphate-free or nitrogen- and phosphate-free half-strength Hoagland's solutions, tap water, or tap water containing 0.4 μ M fluridone. After the plants were grown for 3 days, the culture filtrates were subjected to liquid-liquid partitioning using EtOAc (25 mL $\times$ 4). The EtOAc extracts were combined and evaporated to dryness *in vacuo*, with the residue dissolved in a small amount of hexane and CHCl_3 , roughly purified by silica gel CC (10 $\times$ 6 mm i.d.) with EtOAc in CHCl_3 (10 mL, 30:70, v/v). The residue, dried and dissolved in CH_3CN (100 μ L), was analysed for STLs **2** and **3** and heliolactone (**6**) using LC-MS/MS. UPLC analytical conditions were as follows: column, 100 $\times$ 2.0 mm i.d., 2.5 μ m, COSMOSIL 2.5C₁₈-MS-II, 30 $^\circ\text{C}$; solvent, MeOH in H_2O (50–70% , v/v, 0–20 min, linear gradient); and flow rate, 0.2 mL/min. Compounds **2**, **3**, and **6** were eluted at 19.1 min, 17.6 min, and 10.2 min, respectively. The mass spectrometer was operated in the positive electrospray ionization (ESI) mode with capillary voltage at 3 kV, source temperature at 120 $^\circ\text{C}$, and desolvation gas temperature at 350 $^\circ\text{C}$. The nebulizer and desolvation N_2 gas flows were 50 and 550 L/h, respectively. Fragmentation was performed by collision-induced dissociation (CID) with Ar at 0.1 mL/min. Multiple reaction monitoring (MRM) was used to identify the presence of heliolactone (**6**), DCL (**2**) and costunolide (**3**). MRM transitions were set according to the mass spectra obtained for the authentic samples. The transitions (channels) selected were at m/z 361 > 97 and 361 > 233 for **6**, 231 > 105 and 231 > 187 for **2** and 233 > 91 and 233 > 187 for **3**, the cone voltage was 25 V, and the collision energy was 18 eV.

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Figure legends

Fig. 1. Structures of carlactone (**1**), sesquiterpene lactones (**2** and **3**), strigolactones (**4** and **5**), and reference compounds (**7-10**) use for assignment of heliolactone (**6**).

Fig. 2. The proposed structure of heliolactone (**6**). ^1H - ^1H COSY, major HMBC correlations and observed NOEs are indicated by bold lines, arrows, and double-headed arrows, respectively.

Fig. 3. Effects of heliolactone (**6**) (closed circle), DCL (**2**) (closed square) and costunolide (**3**) (open triangle) on germination of seeds of *S. hermonthica*, *O. cumana*, *O. minor*, *O. crenata*, and *P. aegyptiaca*. Data are shown as mean $\pm$ S.E. ($n=3$). GR24 (1000 nM) (open diamond) was used as a positive control.

Fig. 4. LC-MS/MS analyses of sunflower root exudates. Sunflower seedlings were hydroponically grown in (A) tap water (-P, -N), (B) tap water supplemented with 0.53 mM Na_2HPO_4 (+P, -N), (C) tap water supplemented with 1 mM NH_4NO_3 (-P, +N) or (D) modified 40% Long Ashton nutrient solution (+P, +N). LC-MS conditions are described in the Experimental section. These figures are overlaid MRM chromatograms of the transition at m/z 361 > 97 for heliolactone (**6**) and at m/z 233 > 187 for costunolide (**3**) with intensities of 4.33×10^4 .

Tables

Table 1. ¹H NMR Spectroscopic Data for heliolactone (**6**), norsesquiterpene **7**, and alectrol (**5**) in CDCl₃.

Position ^a	6	Position ^a	7^b	Position (part) ^a	5^c
2	2.09 d (16.5 Hz) 2.34 d (16.5 Hz)	2	2.09 d (16.4 Hz) 2.33 d (16.4 Hz)	7 (A) 6 (A)	1.40-1.48 m 1.60-1.72 m
4	5.91 br.s	4	5.91 s	5 (A)	1.86-1.96 m
6	2.57 d (7.5 Hz)	6	2.55 d (9.0 Hz)		
7	6.25 d ^d (7.5 Hz)	7	5.71 dd (15.2, 9.0 Hz)	8b (BC)	5.62 d (7.3 Hz)
8	6.24 s ^e	8	5.62 dd (15.2, 5.5 Hz)	3a (BC)	3.45 ddd (7.3, 2.7, 1.7 Hz)
		9	4.30 m		
10	7.53 s	10	3.50 dd (11.0, 7.4 Hz) 3.69 dd (11.0, 3.5 Hz)	6' (enol ether)	7.46 d (2.7 Hz)
11	6.15 m			2' (D)	6.16 m
12	6.95 m			3' (D)	6.95 m
16	0.96 s	11	0.96 s	9 (A)	1.13 s
17	1.04 s	12	1.03 s	10 (A)	1.16 s
18	1.91 d (1.1 Hz)	13	1.90 d (1.6 Hz)	4 (B)	5.74 s
19-OMe	3.75 s				
20	2.04 dd (1.5, 1.0 Hz)			7' (D) 2'' (AcO)	2.04 m 2.05 s

^a The numbering and the ring position are shown in Figs. 1 and 2. The numbering of heliolactone (**6**) is based on the nomenclature of carotenoids and the biosynthetic pathway of carlactone proposed by Alder et al. (2012).

^b Reported by Greger et al. (2001).

^c Reported by Ueno et al. (2011).

^d ABX pattern, chemical shift shows the value from the inner two peaks because the outer two peaks were not detectable.

^e AB pattern, chemical shift shows the value from the inner peak because the outer peak was not detectable.

Table 2. NMR Spectroscopic Data for heliolactone (**6**) and 5-deoxystriol (**4**) in C₆D₆.

Helio lactone (6)			5-Deoxystriol (4)		
Position ^a	δ _C	δ _H	Position	δ _C	δ _H
1	36.3 (C)		8	32.1 (C)	
2	48.1 (CH ₂)	2.05 d (16.4 Hz)	7	39.2 (CH ₂)	1.17 ddd (13.0, 10.8, 3.2 Hz)
		2.32 d (16.4 Hz)			1.30 ddd (13.0, 6.7, 3.0 Hz)
3	196.8 (C)		6	19.5 (CH ₂)	1.40 m 1.49 m
4	126.4 (CH)	5.93 br.s	5	26.5 (CH ₂)	1.57 dddm (17.5, 7.4, 7.4 Hz)
					1.71 ddd (17.5, 5.1, 5.1 Hz)
5	159.7 (C)		4a	140.7 (C)	
6	57.3 (CH)	2.15 d (9.1 Hz)	8a	140.3 (C)	
7	133.1 (CH)	6.46 dd (15.9, 9.1 Hz)	8b	87.6 (CH)	5.07 dm (7.7 Hz)
8	122.5 (CH)	6.40 d (15.9 Hz)	3a	36.8 (CH)	3.13 dddd (9.1, 7.7, 3.3, 2.6 Hz)
9	112.2 (C)		3	114.7 (C)	
10	152.7 (CH)	7.42 s	6'	150.4 (CH)	7.37 d (2.6 Hz)
11	100.2 (CH)	4.99 dq (1.5, 1.5 Hz)	2'	100.8 (CH)	5.12 m
12	140.4 (CH)	5.64 dq (1.5, 1.5 Hz)	3'	140.5 (CH)	5.70 m
13	135.3 (C)		4'	135.3 (C)	
14	169.6 (C)		5'	169.8 (C)	
16	26.9 (CH ₃)	0.78 s	10	27.8 (CH ₃)	0.99 s
17	27.7 (CH ₃)	0.79 s	9	28.3 (CH ₃)	1.22 s
18	23.1 (CH ₃)	1.49 d (1.3 Hz)	4	41.4 (CH ₂)	2.20 dm (16.5 Hz) 2.36 dd (16.5, 9.1 Hz)
19	166.3 (C)		2	170.7 (C)	
19-OMe	51.2 (CH ₃)	3.37 s			

20	10.1 (CH ₃)	1.31 dd (1.5, 1.5 Hz)	7'	10.2 (CH ₃)	1.32 dd (1.6, 1.6 Hz)
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^a The numbering of heliolactone (**6**) is based on the nomenclature of carotenoids and the biosynthetic pathway of carlactone proposed by Alder et al. (2012).

Fig 1

Heliolactone (6)

Fig 2

Fig 3

Fig 4

Graphical abstract

Heliolactone, a non-sesquiterpene lactone germination stimulant for root parasitic weeds from sunflower

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Heliolactone, a carlactone-type molecule, was isolated from root exudates of sunflower as a non-sesquiterpene lactone germination stimulant for seeds of root parasitic weeds.