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Oviposition stimulants for the cabbage root fly: isolation from cabbage leaves

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Abstract

Two compounds present on the surface of *Brassica oleracea* cv. *botrytis* leaves have been isolated and identified which stimulate very effectively oviposition in the cabbage root fly, *Delia radicum* and which are perceived by a specific receptor neuron in the tarsal sensillum C_5 of the female fly. Activity of extracts and chromatographic fractions were bioassayed, using oviposition experiments and mainly electrophysiological recordings from the C_5 tarsal contact chemoreceptor sensillum of female flies. Spectroscopic data indicate that the main compound is 1,2-dihydro-3-thia-4,10,10b-triaza-cyclopenta[.a.]fluorene-1-carboxylic acid, a novel compound related to *Brassica* phytoalexins like brassicanal C. It is accompanied by its glycine conjugate.

Keywords: *Brassica oleracea* cv. *botrytis*; Cruciferae; *Delia radicum*; Diptera; Anthomyiidae; Insect; Isolation and identification; Electrospray mass spectrometry; NMR spectroscopy; 1,2-Dihydro-3-thia-4,10,10b-triaza-cyclopenta[.a.]fluorene-1-carboxylic acid; Contact-chemoreceptor neurons; Electrophysiology

1. Introduction

Gravid females of *Delia radicum* L. are known to use olfactory (isothiocyanates) and visual cues to locate potential host plants (Roessingh et al., 1992). For the stimulation of oviposition in the soil, the female's contact with the host-plant leaf surface is essential (Städler and Roessingh, 1991). It has been shown that the cabbage leaf surface contains indole glucosinolates stimulating oviposition and that these molecules are recognised by specific receptor cells in the tarsal D_3 and D_4 sensilla (Roessingh et al., 1992). Recently we reported that the two tarsal C_5 sensilla on the ventromedial side near the distal margin of the 5th tarsomere contain a receptor neuron very sensitive (estimated threshold 10^{-11} M) to unknown compound(s) on the surface of host-plant leaves (Roessingh, Städler, Baur, Hurter, & Ramp, 1997). The activity of this neuron was closely correlated with stimulation of oviposition in the sand below treated

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surrogate leaves (Roessingh et al., 1997). This result provided the necessary basis for using electrophysiological recordings as bioassays sensitive enough for the isolation and purification of these compounds (**1** and **2**) that are present in only minute amounts.

2. Results and discussion

2.1. Homogeneity, physical properties and amount of oviposition stimulant

The isolation and purification procedure is detailed in Section 3. After the last purification step, compounds **1** and **2** eluted from the CN-modified silicagel column at 9.5 and 14.5 min with a base to peak ratios of 0.036 and 0.060, respectively. The longer retention time of **2** under reversed phase conditions indicates a lower polarity. Both active substances produced the following absorption: $\lambda_{max}^{MeOH-H_2O-HCO_2H\ (500:500:1)}$ nm: 195, 270 and 345. UV-absorption, using tryptophan as a reference at 280 nm, indicated for **1** a total amount of 52.5 μg and for **2**, 46.9 μg. These values are corrected for molecular weight. Starting from 17 kg of cabbage leaves, the extraction procedure yielded 2.8–3.1 μg kg^{-1}. The extraction efficiency is not considered in this calculation since plant constituents present in much higher concentrations may alter oviposition- and electrophysiological determinations in crude extracts.

2.2. Structure elucidation

2.2.1. Mass spectrometry

Mass spectra of **1** and **2** were obtained by using electrospray (ES) as a soft ionisation technique. For **1**, the negative ion mode of ESMS gave better sensitivity and higher quality spectra than the positive ion mode. Under the chosen declustering conditions, pronounced deprotonated molecules $(M–H)^-$ were observed at *m/z* 270 together with $[M–H–CO_2]^-$ fragments at *m/z* 226, whose facile formation from this precursor ion was confirmed by tandem mass spectrometry. In addition, MSMS indicated a NCS substructure by loss of CS, NCS and the formation of NCS^- from $[M–H–CO_2]^-$ as precursor. In the positive ion mode, $[M+H]^+$ signals were recorded at *m/z* 272, thus confirming the molecular mass $M_r = 271$ for **1**. For **2**, positive ion MS and MSMS spectra were consistent with glycine conjugation of **1**, as an increase of M_r by 57 Da (–$NHCH_2CO$–, $[M+H]^+$ at *m/z* 329) was observed in MS together with losses of H_2O/CO and H_2NCH_2COOH followed by CO in MSMS of *m/z* 329. An accurate mass measurement under high-resolution conditions (resolving power 5000) yielded a mass value of 329.0705 for this ion. This was in excellent agreement (error of 1 ppm) with the theoretical value calculated for the chemical composition $C_{15}H_{12}N_4O_3S$ of **2** and, hence, $C_{13}H_9N_3O_2S$ of **1**.

2.2.2. NMR spectroscopy

The structure of **1** is given in Fig. 1(a) together with the ^{13}C-NMR chemical shifts obtained from HSQC and HMBC experiments. The ^{1}H-NMR spectral data are given in Section 3. The HMBC data have been measured of material isolated from rutabaga roots (*Brassica napus* var. *napobrassica*), since isolation from the roots yielded about ten times more material then isolation from the leaves (De Jong et al., unpublished). The identity of the compounds isolated from either source was ascertained by ^{1}H-NMR and mass spectrometry as well as by HPLC.

Fig. 1(b) shows the HMBC correlations used for the structure elucidation. These HMBC correlations firmly establish the connection between the indole ring system and the pyrimidine ring. The 10,10a double bond (for the numbering cf. Fig. 2) is supported by the carbon chemical shifts of the phenyl ring (especially the value of 153.8 ppm for the carbon carrying the indole N atom) as compared with similar compounds (cf. e.g. Adam & Wonkler, 1984). In addition, an NOE experiment showed that the pyrimidine proton (8.78 ppm) is in close vicinity to the aromatic proton at 8.04 ppm. No NOEs were observed between the aliphatic and the aromatic protons of the molecule. Likewise, no benzylic coupling between the CH_2CH group and the aryl protons is observed excluding a direct attachment

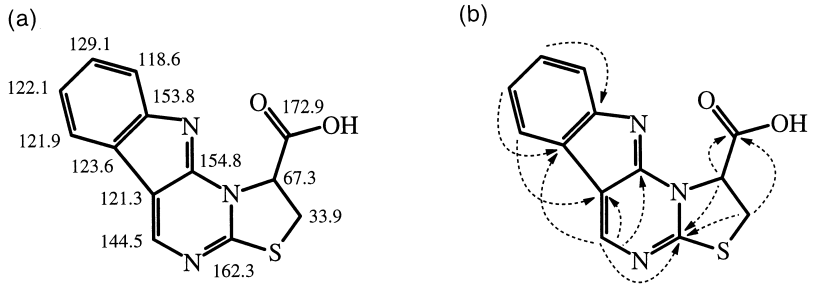

Fig. 1. Compound **1**: (a) ^{13}C-NMR chemical shifts in CD_3OD; (b) HMBC correlations used for the structure elucidation.

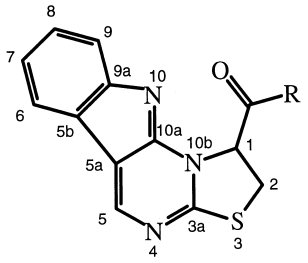

Fig. 2. Compound **1**: R = OH, compound **2**: R = $NHCH_2COOH$.

of the CH_2CH group to the phenyl ring. The HMBC correlations to the C atom at 162.3 ppm from both the pyrimidine proton and the CH_2CH group indicated that the CH_2CH group is annellated to the pyrimidine ring. The CH_2CH group is part of a thiazoline ring as shown by the similarity of its ^{1}H-NMR spectral data with that of the thiazoline ring in reference compound **3** (Cherbuliez, Espejo, Willhalm, & Rabinowitz, 1968; Sauter, Fröhlich, Blasl, & Gewald, 1995) (cf. Fig. 3) and the large $^1J_{CH}$ coupling of the CH_2 protons (150 and 152 Hz, respectively, as measured by an HSQC experiment without proton decoupling during the acquisition).

An alternative structure could be drawn for **1**, in which the thiazoline ring is closed with N(4) instead of N(10b) (cf. Fig. 2). The fact that no NOE and no HMBC correlation were observed between the pyrimidine H and the CH_2CH group excludes this alternative structure. It is also excluded by the chemical shift of H-1 of **1** (5.82 ppm), which appears at an even lower field than the analogous proton in **3** (5.50 ppm Cherbuliez et al., 1968). These values are to be compared, for example, with that of 4.6 ppm for the alpha H in proline in random coil proteins (Wüthrich, 1976). The reason for the strong down field shift in **1** or **3** is the deshielding caused by the aryl C=N or the C=O double bond in *peri* position to the bond C(1)–H. This *peri* arrangement is absent in the alternative structure, resulting in an expected absorption at higher field for H-1 then that observed.

Compound **2** (Fig. 2) is a conjugate of **1** with the amino acid glycine as documented by the AB system at 4.01 and 3.63 ppm as well as the mass spectral data given above. Both compounds are novel and related to *Brassica* phytoalexins like brassicanal C (Monde, Sasaki, Shirata, & Takasugi, 1991). Indeed, the formation of **1** in the plant can be envisaged as a condensation of the precursor of brassicanal C and 2-aminothiazoline-4-carboxylic acid, formed by cyanide and cystine (common detoxification product of cyanide (see, for example, Weuffen, Jess, Juelich, & Bernhardt, 1980; Kawakishi, Goto, & Namiki, 1983 and literature cited therein). Thus, it does not seem incidental that the *Brassica* phytoalexins have also been shown to be active as oviposition stimulants for the cabbage root fly *Delia radicum* (Baur, Städler, Monde, & Takasugi, 1998).

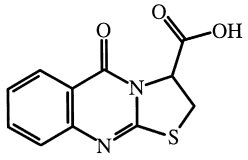

Fig. 3. Reference compound **3**.

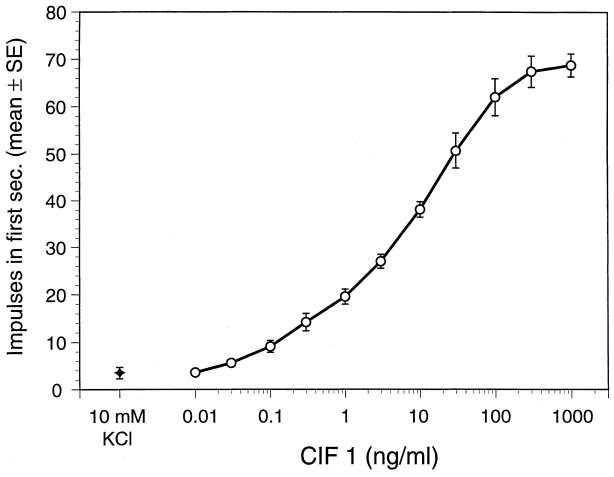

Fig. 4. Dose–response curve of compound **1**. Response of C_5-sensillum on the foretarsi of *Delia radicum* females to compound **1**, dissolved in 10 mM KCl. Response measured as number of impulses in first second after onset of stimulation. Number of sensilla tested for each concentration was between 7 and 11.

For any isolation and identification of active compounds, biological assays are of paramount importance. In this study we depended heavily on electrophysiological bioassays, which proved to be time-saving, highly sensitive and selective, thus requiring minimal amounts of test material. Since electrophysiological recordings (spike counts) alone cannot reveal the function of these signals in behaviour, an excellent correlation of the spike counts to the oviposition assay was essential (Roessingh et al., 1997). Based on the dilution experiment represented in Fig. 4 and a molecular weight of 271 for **1**, we estimate a kb-value of 3.7×10^{-8} M and a threshold of 3.7×10^{-11} M. This is very low and comparable to the response of the so far most sensitive contact chemoreceptors specific for the host marking pheromone of the cherry fruit fly *Rhagoletis cerasi* L. (Städler, Ernst, Hurter, & Boller, 1994).

3. Experimental

3.1. Plant material and devices

Brassica oleracea L. cv. *botrytis* CC Cross were

grown in the field to the 8–10 leaf stage at Wädenswil. All leaves were harvested at the same time and subjected to the extraction procedure. For grinding of the leaf material we used two mills: Lochscheibenmühle (ML 150–300) and Kolloidmühle (MZ 170–180). The chromatographic system consisted of a multisolvent delivery system and a 990 photodiode array detector, permitting spectrum analyses from 190–800 nm.

3.2. Insects and bioassays

The flies originated from the laboratory culture maintained continuously in the laboratory at Wädenswil (Roessingh et al., 1992). This culture was started from field-collected material in 1991. The oviposition assay and the electrophysiological recording technique have been described in detail in Roessingh et al. (1992). The type of surrogate leaf was identical to the earlier model, but reduced in size to: 7×7 cm surface projection and a flat stem of 5×2 cm.

3.3. Extraction- and purification procedure

For the initial extraction of the biologically active material, leaf-surface- and total-leaf extractions were used (Roessingh et al., 1997). The latter turned out to be superior, since it yielded a higher electrophysiological activity.

3.4. Leaf-surface extraction

2 kg of whole leaves were surface extracted at room temperature by dipping each time for 5 s in the following solvents: 2.0 l of CH_2Cl_2, 5.0 l of MeOH and again 5.0 l of MeOH. The combined methanol extracts were filtered through filter paper on a Büchner funnel of 270 mm i.d. and concentrated to approximately 100 ml on a rotation evaporator (45°C, 150 mbar). This procedure was repeated 18 times (36 kg of leaves). The evaporated solvents were re-used for subsequent extractions. The recombined residues were diluted with MeOH–H_2O(1:1) to 8.0 l and subjected, in one portion, to ion exchanging chromatography.

3.5. Total-leaf extract

15 kg of whole leaves were frozen in liquid N_2, crushed in a mortar to pieces of 0.5–10 mm and stored at −20°C. For homogenisation, this plant material was poured, under frozen conditions, into 75 l of MeOH–H_2O(1:1). The suspension was ground successively with the Lochscheibenmühle and the Kolloidmühle at ambient temperature. The resulting slurry was filtered on a nylon net (mesh 300 μm) followed by a Büchner funnel of 270 mm i.d. equipped with a filter paper Macherey-Nagel 604. The filtrate was kept overnight at room temperature and thereupon processed by ion exchanging chromatography in 3 portions of 25 l each.

3.6. Ion exchanging chromatography

The crude extract was purified successively on two different resins. (1) Column: Dowex 50W X4 50/100 mesh, H^+-form; bed size: 70 mm i.d. × 400 mm. All solvents for conditioning, elution and regeneration, were made of MeOH–H_2O (1:1). Leaf-surface or total-leaf extracts (8 or 25 l, respectively, of MeOH–H_2O (1:1)) were applied to the column with a peristaltic pump at a flow rate of 85 ml min^{-1}. The same device was used for the elution process. Elution took place as follows: 1.0 l of MeOH–H_2O (1:1), 3.6 l of 1 M aqueous NH_3 and 2.5 l of 2 M aqueous NH_3. Electrophysiologically active compounds appeared with 1 M aqueous NH_3. It was evaporated and the residue dissolved in 200 ml of H_2O (4 batches). (2) Column: BioRad AG 50 W X16 200–400 mesh NH_4^+-form; bed size: 36 mm i.d. × 300 mm; injected volume: 40 ml of active solution in H_2O out of 800 ml (20 repetitions); solvents for elution: (A) 0.01 M aqueous NH_3 buffered with HCO_2H to pH 3.5, (B) 0.5 M aqueous NH_3 buffered with HCO_2H to pH 6.4 and (C) 1.0 M aqueous NH_3. Elution programme: 0–5 min A, 5–30 min linearly from A to B, 30–40 min B, 40–140 min C (step) at a flow rate of 10 ml min^{-1}. Electrophysiologically active compounds appeared between 55 and 125 min. Solvents were evaporated and residues dissolved in 30 ml of CH_3CN–H_2O (1:9).

3.7. MPLC (medium pressure liquid chromatography)

Column: CN-modified silica gel with 36 mm i.d. × 460 mm; material: RSiL CN 15–35 μm; filling procedure: dry powder technique (Talamona & Stump, 1985); injection volume: 5.0 ml of active solution in CH_3CN–H_2O (1:9) out of 30 ml (6 repetitions). Elution occurred on a binary solvent system, A: H_2O, B: CH_3CN–HCO_2H (1000:1). Program: 0–10 min A; 10–15 min 10% B in A (step); 15–60 min linearly from 10% B in A to 100% B; 60–90 min 100% B. Flow rate: 20 ml min^{-1}. This procedure resulted in 45 fractions of 2 min each (40 ml). Electrophysiologically active regions were in fractions 16–21. The solvent was evaporated to dryness and the residue was dissolved in 2 ml of CH_3CN–H_2O(1:3).

3.8. HPLC on CN-modified silica gel (preparative)

Column: 10 mm i.d. × 250 mm; material: Nucleosil 100 CN, 7 μm (MN); pre-column of 4 mm i.d. × 20 mm of the coarser adsorbent (30–40 μm); injected volume: 0.4 ml of active solution in CH_3CN–H_2O (1:3) out of 12 ml (30 repetitions) of the preceding purification

step. Elution was performed on a binary solvent system consisting of A: H_2O–CH_3CN (99:1) and B: CH_3CN–HCO_2H (1000:1). The column was preconditioned as follows: programme: 0–15 min linear gradient from 3% B in A to 10% B in A; 15–16 min linearly from 10% B to 20% B in A; 16–21 min 20% B in A isocratically with a flow rate of 6.0 ml min^{-1}. 42 fractions (3.0 ml) were collected and the electrophysiological activity (nerve impulses) of the same receptor neuron appeared in two active regions: activity 1 in fraction 11–13, activity 2 in fraction 14–15. The active regions were collected separately, the solvents evaporated and the residues taken up in 5.0 and 2.0 ml, respectively of H_2O–MeOH (9:1).

3.9. HPLC on phenyl-modified silicagel (activity 1 and 2)

Column: 10 mm i.d. × 250 mm; material: Nucleosil 100 C_6H_5, 7 μm; injected volume: 0.5 ml out of 5 ml (10 repetitions) of the previous purification step; elution solvents: A: H_2O–CH_3CN (99:1) and B: MeOH. Programme: 0–1 min A, 1–25 min linearly from A to B, 25–35 min B. Flow rate: 6.0 ml min^{-1}. 40 fractions (3.0 ml) were collected and the electrophysiologically active compounds eluted in fraction 18–20. Solvents were evaporated to dryness and redissolved in 0.5 ml of H_2O. For the activity 2 the same procedure as for activity 1 was applied with 4 repetitions and electrophysiological activity was found in fractions 17–20.

3.10. HPLC on CN-modified silicagel (analytical)

Activity 1 and 2 were processed further by the same procedure: Column: 4 mm i.d. × 250 mm; material: Nucleosil 100 CN, 10 μm protected by a precolumn of 4 mm i.d. × 30 mm containing the same adsorbent; injected volume: 50 μl of the previous purification step in H_2O out of 500 μl were injected (10 repetitions). A binary solvent system for elution was used: (A) H_2O–CH_3CN (99:1) and (B) MeOH–HCO_2H (1000:1). Programme: 0–15 min linearly 3% B in A to 20% B in A, 15–25 min 20% B in A linearly to 100% B at a flow rate of 1.0 ml min^{-1}. 50 fractions (0.5 ml) were collected and the electrophysiological activity appeared in fractions 17–20 for activity 1 and 28–31 for activity 2. Both solutions were lyophilised separately to obtain yellowish powders. These preparations (not weighed) were subjected to structural investigations.

3.11. NMR spectroscopy

The NMR spectra were measured with a Varian Unity 500 NMR spectrometer in CD_3OD at 25°C. Signal assignments are based upon COSY, HSQC, HMBC and NOE experiments.

3.12. Compound **1** (1,2-dihydro-3-thia-4,10,10b-triazacyclopenta[.a.]fluorene-1-carboxylic acid)

^{1}H-NMR (CD_3OD, $^1J_{CH}$ as obtained from an HSQC experiment without proton decoupling during the acquisition): 8.78 (s, H-5, $^1J_{CH}=184$); 8.04 (db, $J=8$, H-6); 7.61 (db, $J=8$, H-9); 7.45 (td, $J=8$ and 1.5, H-8); 7.24 (td, $J=8$ and 1, H-7); 5.82 (ddb, $J=8.5$ and 1.2, H-1, $^1J_{CH}=150$); 4.16 (dd, $J=11.5$ and 8.5, H-2, $^1J_{CH}=152$); 3.94 (dd, $J=11.5$ and 1.2, H-2, $^1J_{CH}=150$). ^{13}C-NMR chemical shifts cf. Fig. 1a.

3.13. Compound **2**

^{1}H-NMR (CD_3OD): 8.80 (s, H-5); 8.04 (db, $J=8$, H-6); 7.60 (db, $J=8$, H-9); 7.46 (td, $J=8$ and 1.5, H-8); 7.25 (td, $J=8$ and 1, H-7); 6.04 (ddb, $J=8.5$ and 1.2, H-1); 4.22 (dd, $J=11.5$ and 8.5, H-2); 4.05 (dd, $J=11.5$ and 1.2, H-2); 4.01 and 3.63 (AB system, $J=17$, NCH_2CO).

3.14. Mass spectrometry

Low-resolution MS analyses were carried out using a PE Sciex API-III triple-quadrupole mass spectrometer operated under standard electrospray ionisation conditions in positive or negative ion mode as stated. For MSMS experiments, argon was chosen as target gas. Accurate mass measurement under high resolution conditions was performed using a Finnigan MAT 900 S sector instrument equipped with an electrospray interface. Instrumental resolution was adjusted to 5000 and the peak matching technique was used for mass determination based on $(M+Na)^+$ adducts of polypropylene glycol as reference ions. Compound **1**: ESMS: full spectrum: *m/z* 270, $[M–H]^–$; *m/z* 226, $[M–H–CO_2]^–$; MSMS of *m/z* 270: *m/z* 226, $[M–H–CO_2]^–$; *m/z* 200 [*m/z* 226-CN./C_2H_2]; *m/z* 182, [*m/z* 226-CS]$^–$; *m/z* 168 [*m/z* 226-NCS.]$^–$; *m/z* 166 [*m/z* 200- $H_2S]^–$. MSMS of *m/z* 226: *m/z* 200 [*m/z* 226-CN./$C_2H_2]^–$; *m/z* 182, [*m/z* 226-CS]$^–$; *m/z* 168 [*m/z* 226-NCS.]$^–$; *m/z* 166 [*m/z* 200-$H_2S]^–$; *m/z* 58, $NCS^–$, *m/z* 26, $CN^–$. +ESMS: *m/z* 272, $[M+H]^+$; *m/z* 294, $[M+Na]^+$, *m/z* 565, $[2M+Na]^+$. Compound **2**: +ESMS: *m/z* 329, $[M+H]^+$; MSMS of *m/z* 329: *m/z* 283, $[M+H –H_2O–CO]^+$; *m/z* 254, $[M+H – H_2NCH_2COOH]^+$; *m/z* 226, [*m/z* 254-CO]$^+$; *m/z* 168, [*m/z* 226 -NCS.]$^+$.

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