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Specific accumulation and revised structures of acridone alkaloid glucosides in the tips of transformed roots of *Ruta graveolens*

Inna Kuzovkina[a,*], Irina Al'terman[a], Bernd Schneider[b]

[a] *Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya ul. 35, 127276 Moscow, Russia*
[b] *Max-Planck-Institute for Chemical Ecology, Beutenberg Campus, Hans-Knöll-Str. 8, D-07745 Jena, Germany*

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Dedicated to Professor Detlef Gröger on the occasion of his 75th birthday

Abstract

The root tips of *Ruta graveolens* (common rue) show strong autofluorescence of acridone alkaloids, which are characteristic secondary metabolites of this plant. To study the specific distribution and accumulation of acridone alkaloids in various root segments of *Ruta graveolens*, root material was harvested from genetically transformed root cultures and extracts were investigated by chromatographic techniques and HPLC-^{1}H NMR spectroscopy. The cells of the elongation and differentiation zones contained acridone glucosides and large amounts of acridone alkaloids, mainly rutacridone. Gravacridondiol glucoside was identified as the dominant secondary compound of the root tips and its structure revised by means of spectroscopic methods. In addition, minor acridones, including the structurally revised gravacridontriol glucoside and unknown natural products, were found in the root tip.

Keywords: *Ruta graveolens*; Rutaceae; Acridone alkaloids; Acridone glucosides; Accumulation; Gravacridondiol glucoside; Hairy root culture; HPLC-NMR; Root tips; Rutacridone

1. Introduction

Ruta graveolens L. (common rue) is a medicinal plant whose roots and aerial parts contain more than 120 compounds of different classes of natural products such as acridone alkaloids, coumarines, essential oils, flavonoids, and furoquinolines (Sprecher, 1956; Gröger 1988; De Feo et al., 2002; Oliva et al., 2003). Many of these compounds are physiologically active and therefore of pharmacological interest. The high level of secondary compounds in *Ruta* plants encouraged several groups to establish in vitro cell and root cultures (Reinhard et al., 1968; Kuzovkina et al., 1971, 1979; Ramawat et al., 1985) in order to investigate biosynthetic pathways (Baumert et al., 1982; Maier et al., 1990) and key enzymes (Kuzovkina et al., 1987; Baumert et al., 1994). The intense autofluorescence of some compounds from *Ruta* allows them to be easily localized in the tissue (Versar-Petri et al., 1976; Zobel and Brown, 1989). Distribution and compartmentalization studies revealed, for example, the accumulation of acridone alkaloids in roots of intact *Ruta* plants (Rozsa et al., 1981). Thus, although acridone alkaloids can be considered root-specific natural products, they are produced in dedifferentiated heterotrophically grown cell cultures as well (Scharlemann, 1972; Kuzovkina et al., 1980; Eilert et al., 1984). There is little information about the distribution of acridone alkaloids within roots of intact plants (Kuzovkina et al., 1975; Eilert et al., 1986; Junghanns et al., 1998). Localization in cells of the rhizodermis and ray parenchyma hints at a radial, outwardly directed transport process. Versar-Petri et al. (1976) reported specific fluorescence of acridone alkaloids in root tips, which was confirmed by later observations (Kuzovkina et al., 1980). However, due to similar UV and fluorescence spectra, investigation of the distribution of specific acridone alkaloids in the root tissue is hardly possible by absorption or fluorescence microscopic and spectroscopic techniques without extraction and chromatographic separation. To understand the distribution

* Corresponding author. Tel.: +007-095-977-9211; fax: +007-095-977-8018.
E-mail address: ikuz@ippras.ru (I. Kuzovkina).

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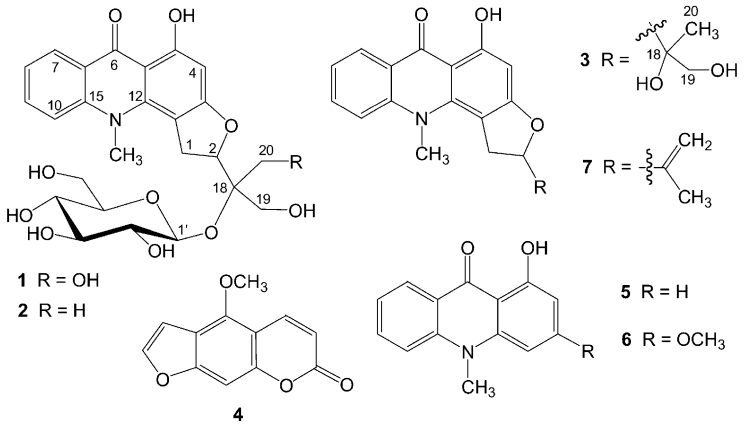

and accumulation of specific acridone alkaloids in the root tip of *Ruta graveolens* and their identification, root tip material was obtained from genetically transformed root cultures and extracts were investigated by chromatographic techniques and HPLC-^{1}H NMR.

2. Results

HPLC-Analysis of the MeOH extracts of whole roots of the *Ruta graveolens* hairy root culture showed numerous UV-detectable compounds (Fig. 1A). The major peaks in the extracts were assignable to acridone alkaloids and coumarines by means of their UV spectra. The characteristic broad UV absorption band with a maximum at about 405 nm was used to differentiate between acridone-type compounds and other components of the *Ruta* extracts. In addition to the absorption properties, which are very similar to all acridone alkaloids, individual compounds of that type were identified by comparing their retention times with those of authentic reference compounds. Using this strategy, some of the major compounds of the MeOH extract of whole roots (Fig. 1A) have been identified as acridone glucosides (**1**, R_t 20.8 min; **2**, R_t 21.6 min), gravacridondiol (**3**, R_t 22.9 min), 1-hydroxy-*N*-methylacridone (**5**, R_t 28.2 min), and 1-hydroxy-3-methoxy-*N*-methylacridone (**6**, R_t 28.7 min) and the major alkaloid of *Ruta* roots, rutacridone (**7**, R_t 32.2 min). Compounds of other classes of natural products such as the furocoumarin bergapten (**4**, R_t 25.9 min) were also detected. Based on the peak areas in the UV profiles recorded at 275 nm (Fig. 1A) and 405 nm (Fig. 1B) of the MeOH extract of whole roots, the levels of the acridone glucoside (**2**) and rutacridone (**7**) are in the same order of magnitude. Compounds **1**, **3**, **5**, **6** and a number of minor unidentified acridones, detectable both at 275 and 405 nm, are less abundant.

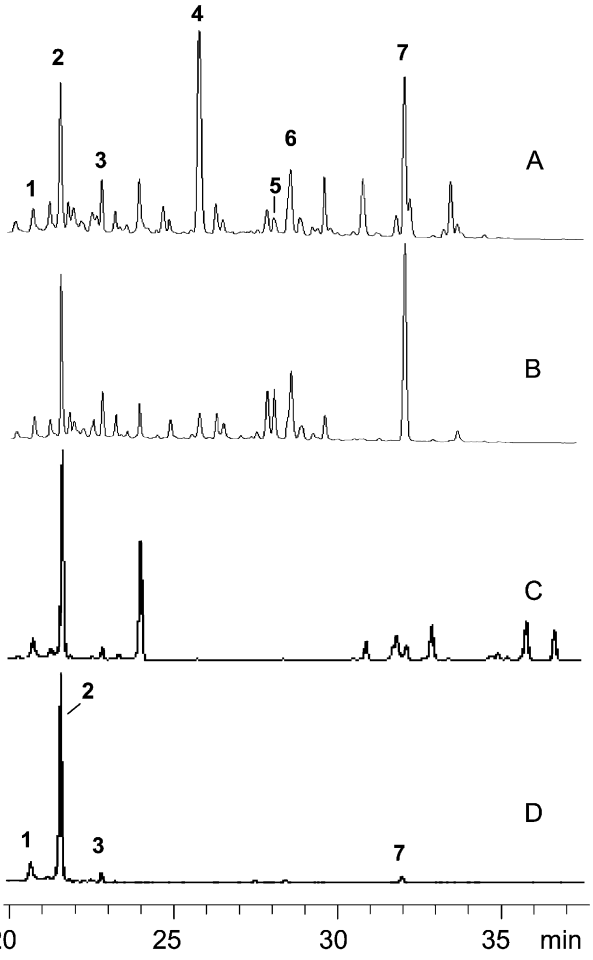

Fig. 1. HPLC profiles of MeOH extracts of *Ruta graveolens* hairy roots. A: Whole roots, 275 nm. B: Whole roots, 405 nm. C: Root tips (apical meristeme and calyptra), 275 nm. D: Root tips (apical meristeme and calyptra) 405 nm. Gravacridontriol-18-β-D-glucoside (**1**), gravacridondiol-18-β-D-glucoside (**2**), gravacridondiol (**3**), bergapten (**4**), 1-hydroxy-*N*-methyl-rutacridone (**5**), 1-hydroxy-3-methoxy-*N*-methyl-rutacridone (**6**), and rutacridone (**7**) were identified as described in the text.

The UV profiles of the *n*-hexane, $CHCl_3$, and EtOAc fractions of the whole roots and different root sections showed enhanced concentrations of certain acridone alkaloids and other secondary products compared to the MeOH extract. Comparison with the retention time of reference compounds confirmed the occurrence of rutacridone (**7**), 1-hydroxy-*N*-methylacridone (**5**), 1-hydroxy-3-methoxy-*N*-methylacridone (**6**), and gravacridondiol (**3**) in the $CHCl_3$ extract. Other compounds remained unidentified since the retention time did not agree with any of the available reference compounds.

The fractions obtained from different root segments and those from the whole root revealed substantial differences in the metabolite pattern. The most lipophilic acridone alkaloid detected in this study, rutacridone (**7**), was found in the differentiation zone where the root hairs are located, suggesting the compound accumulates in the trichomes. More hydrophilic acridones such as **3** and **6** and other metabolites are depleted in this root segment. In contrast, the pattern of the EtOAc extract, which represents more hydrophilic compounds, did not show essential differences between the whole roots, the cell elongation zone, and the differentiation zone. The acridone glucoside (**2**) is clearly the dominant component in these fractions.

The MeOH extract of the root tip (apical meristem and calyptra; 9.2 mg dry extract matter only) was not solvent-partitioned but analyzed directly by TLC and HPLC. Using solvent systems 1 and 2, TLC showed only two UV detectable spots of an unpolar fraction (R_f 0.8 in solvent system 1) and one hydrophilic spot (R_f 0.55 in solvent system 2), corresponding to a glucosidic fraction. The HPLC profile recorded at 275 nm (Fig. 1C) showed a large peak of acridone glucoside (**2**), another significant peak of an unknown compound (R_t 24.0 min), several peaks of unidentified lipophilic compounds (R_t 31–37 min), and a minor amount of rutacridone (**7**). UV detection at 405 nm exhibited only one dominant peak at R_t 21.6 min (**2**; Fig. 1D) and very minor peaks of acridone-type compounds **1**, **3** and **7**. Due to its single strong UV absorption band at 270 nm and missing absorption at longer wavelength, the peak at R_t 24.0 min was ruled out as an acridone and other known compound from *Ruta graveolens*. The same is true for the lipophilic peaks, which show weak absorption maxima at 282 nm.

Table 1
^{1}H NMR and ^{13}C NMR chemical shifts (δ relative to TMS), coupling constants (J in Hz), and selected HMBC correlations of acridone glucosides **1** and **2** from hairy root cultures of *Ruta graveolens*. Samples were measured in MeOH-d_4 at 500 MHz (^{1}H)

Position	**1**			**2**		
	δ ^{1}H (J in Hz)	δ ^{13}C	HMBC	δ ^{1}H (J in Hz)	δ ^{13}C	HMBC
1	3.74 *dd* (J = 9.5, 14.6)	33.5	2, 16, 17, 18	3.74 *dd* (J = 9.1, 14.7)	34.4	2, 16, 18
	4.17 *dd* (J = 8.8, 14.6)			3.95 *dd* (J = 8.7, 14.7)		
2	5.16 *t* (J = 9.5)	86.2	19, 20	5.02 *t* (J = 9.1)	88.5	
4	6.18 *s*	93.4	5, 13, 16, 17	6.16 *s*	93.4	5, 13, 16, 17
5		166.8			166.8	
6		182.8			182.5	
7	8.30 *d* (J = 7.9)	127.1	6, 9, 15	8.30 *d* (J = 7.9)	127.3	6, 9, 15
8	7.31 *dd* (J = 7.9, 8.5)	123.0	10, 14	7.31 *dd* (J = 7.9, 8.5)	123.1	10, 14
9	7.79 *dd* (J = 8.5, 8.5)	135.7	7, 15	7.79 *dd* (J = 8.5, 8.5)	135.8	7, 15
10	7.65 *d* (J = 8.5)	116.9	8, 14	7.64 *d* (J = 8.5)	116.9	8, 14
12		144.4			144.0	
13		107.6			107.4	
14		122.5			122.3	
15		145.8			145.4	
16		103.3			103.1	
17		168.4			168.9	
18		83.0			81.7	
19	4.01 *d* (J = 12.3)	62.2	2, 18, 20	3.85 *d* (J = 11.6)	65.9	2, 18, 20
	3.88 *d* (J = 12.3)			3.69 *d* (J = 11.6)		
20	3.87 *d* (J = 12.0)	62.7	2, 18, 19	1.39 *s*	17.8	2, 18, 19
	3.86 *d* (J = 12.0)					
N-CH_3	4.06 *s*	39.2	12,15	4.06 *s*	39.3	12, 15
1′	4.88 *d* (J = 7.8)	99.7	18	4.70 *d* (J = 7.8)	99.3	18
2′	3.19 *dd* (J = 8.0, 9.2)	75.5	3′	3.20 *dd* (J = 8.0, 9.2)	75.7	1′, 3′
3′	3.27[a]	78.3		3.35[§]	78.1	2′, 4′
4′	3.27[a]	71.7		3.30[§]	71.6	3′
5′	3.27[a]	78.3		3.42[§]	79.1	
6′	3.77 *d* (J = 11.7)	63.0		3.89 *d* (J = 12.0)	63.0	
	3.64 *dd* (J = 11.7, 5.3)		5′	3.64 *dd* (J = 12.0, 5.1)		5′

[a] Overlayed by residual signal of MeOD, δ values obtained from HMBC and HMQC spectra.

Because peak **2** dominated the HPLC profile of the root tip, its unambiguous identification was a focus of this study. The two known acridone alkaloid glucosides in *Ruta*, first isolated by Reisch et al. (1976) as a mixture, were shown to contain gravacridondiol and gravacridontriol as aglycones and glucose as the conjugating moiety. In order to obtain pure glucosides as reference compounds, the original glucoside mixture of Reisch and collaborators was separated by TLC, purified by preparative HPLC, and subjected to MS and NMR analysis. Spectroscopic data confirmed compound **1** (ESI-MS: m/z 520 $[M+H]^+$) as a glucoside of gravacridontriol and compound **2** (ESI-MS: m/z 504 $[M+H]^+$) as a glucoside of gravacridondiol (**3**). HMBC spectra of both compounds (Table 1) exhibited correlations of the anomeric H-1′ ($^3J=7.8$ Hz) of the β-glucosyl unit with C-18, indicating attachment to the hydroxyl group at C-18 and not at C-19, as previously reported (Reisch et al., 1976). Thus, the structures of the glucosides have to be revised as gravacridondiol-*O*-18-β-D-glucoside (**2**) and gravacridontriol-*O*-18-β-D-glucoside (**1**). The R_f (TLC) and R_t (HPLC) values of both glucosides **1** and **2** were identical with those of the corresponding compounds from transformed root cultures. Moreover, HPLC-^{1}H NMR spectra of glucosides **1** and **2** from the root tip extract were compared with those obtained from the authentic reference samples under identical conditions. The identity of the spectra, in addition to retention times and UV spectra recorded online by DAD, proved that the dominant compound in the extracts of the root tip was gravacridondiol-*O*-18-β-D-glucoside (**2**) and the minor glucosidic component was gravacridontriol-*O*-18-β-D-glucoside (**1**).

3. Discussion

Nearly 20 acridone alkaloids have been isolated so far from intact plants and from various cell and root cultures of *Ruta graveolens* (Szendrei et al., 1976; Rozsa et al., 1976, 1981; Eilert et al., 1982; Gröger, 1988; Paulini et al., 1991). Several cell and root culture lines were established in our laboratory (Kuzovkina et al., 1979, 1980; Kuzovkina, 1992) and, in addition to phytochemical studies, used for investigations of biosynthetic pathways and enzymes. Acridone synthase, the key enzyme of this pathway, was isolated from elicited cell suspension cultures (Baumert et al., 1994), cloned, heterologously expressed, and identified as a member of the plant-specific polyketide synthase family (Junghanns et al., 1995). The biosynthetic steps between rutacridone and glucosides **1** and **2** are still hypothetical. A pathway postulated by Rozsa et al. (1976) involving epoxidation, epoxide ring opening, and subsequent glucosidation is supported by the common occurrence of the corresponding epoxides and gravacridone type aglycones and glucosides in *Ruta* (Rozsa et al., 1981; Nahrstedt et al., 1981; Eilert et al., 1982). Glucosides **1** and **2**, which could be considered final metabolic products, were first isolated as a mixture (Reisch et al., 1976). Hydrolysis of this mixture resulted in gravacridondiol, gravacridontriol, and glucose. Attachment of the glucose to the primary hydroxyl was deduced from the different reactivities of the primary and tertiary hydroxyl groups during acetylation. The IR spectrum of the acetylated glucoside mixture exhibited a vibration band at 3300 cm^{-1} typical of a free alcoholic hydroxyl group. Thus, the acetylated glucoside should still have had a free tertiary hydroxyl remaining and the glucose unit attached to a primary hydroxyl. The authors supported this finding with ^{1}H NMR chemical shifts of two primary *O*-acetyl groups (δ 2.10 and 2.13) of diacetylated gravacridontriol. ^{13}C NMR data (Bergenthal et al., 1979) confirmed the glucosidic structure of gravacridondiol and -triol but were not suitable to prove the glucosidation site. Our NMR analysis, especially HMBC studies, of glucosides **1** and **2** led to revised structures of both glucosides with the glucose attached to the tertiary hydroxyl groups.

The role of rutacridone alkaloids *in planta* is still not completely understood. While most of the acridone alkaloids, e.g. rutacridone and gravacridondiol, were shown to occur constitutively in roots and cell cultures of *Ruta graveolens*, some compound are inducible. The acridone epoxides, which show substantial antimicrobial activity, are of special interest because of their typical phytoalexin properties, e.g. their ability to be induced upon elicitor treatment (Eilert et al., 1984). The present investigation has demonstrated specific accumulation of acridone glucosides in root cultures of *Ruta graveolens* transformed by pRi T-DNA. Gravacridondiol glucoside was shown to be the major compound in the root tips. From these HPLC studies it is not yet clear whether accumulation takes place in the meristematic tissue or in the calyptra cells. Histochemical studies using fluorescense microscopy and microspectral photometry are required to investigate tissue- or cell-specific distribution in more detail.

It should be noted that coloured root tips have been observed already earlier. Molisch (1928) was the first to report on red root tips, presumably due to anthocyan formation, of plants of the Crassulaceae, Saxifragaceae, and some other families. Brigham et al. (1999) showed the occurrence of naphthoquinones in root tips of *Lithospermum erythrorhizon* hairy roots. Recently Opitz et al. (2003) demonstrated that fluorescent phenylphenalenone-type compounds accumulated tissue-specific in the apical meristem and the calyptra of roots of *Xiphidium caeruleum* (Haemodoraceae). The field of root-specific synthesis, accumulation and secretion of secondary plant products is rapidly developing and interesting results can be expected in the near future

(Hawes et al., 2000; Bais et al., 2001; Walker et al., 2003).

4. Experimental

4.1. Plant material

Genetically transformed root cultures of *Ruta graveolens* L. (common rue), which were established from *Ruta* seedlings by treatment of the hypocotyl with *Agrobacterium rhizogenes* (wild strain 15834) (Kuzovkina, 1992), were used in this study. The pRi T-DNA transformed roots were grown in liquid B5 medium (Gamborg et al., 1968) without hormones at 26 °C in the dark on rotary shakers (90 rpm). Subculturing of about 500 mg root material (fw) was performed every 4 weeks into 50 ml of fresh medium. Roots were harvested from 3-week-old cultures and subdivided into root tip (calyptra and meristematic zone, 2–3 mm), zone of cell elongation (5 mm), and differentiation zone (10 mm). In total, segments from 800 roots were collected under a stereo microscope (magnification×16). The root segments were immediately frozen with liquid N_2 before extraction. In addition to separated root segments, intact roots of the transformed root culture were used as control samples.

4.2. Extraction

Freshly collected roots and root segments were homogenized and exhaustively extracted with MeOH in an ultrasonic bath at 40 °C (4×20 min). The pooled extracts were filtered and evaporated. A dry matter of 9.2 mg extract was obtained from the meristematic zone (2 g fresh weight, corresponding to approx. 100 mg dry weight) and directly subjected to TLC and HPLC analysis. Prior to analysis, the methanolic extracts obtained from the zones of cell elongation and differentiation and from intact roots were partitioned between *n*-hexane–H_2O, $CHCl_3$–H_2O, and EtOAc–H_2O. For comparison, aliquots of the methanolic extract were used for HPLC analysis without partitioning. Reference compounds were from Prof. Reisch (Münster), Prof. Szendrei (Szeged), Dr. Baumert (Halle) and our own collection.

4.3. Chromatographic methods

Plant extracts and isolated compounds were analyzed by thin layer chromatography on Merck silica gel sheets $60F_{254}$. Preparative separation of acridone alkaloids was performed using precoated TLC plates (Merck silica gel $60F_{254}$; 20×20 mm; 0.25 mm thickness). Acridone alkaloids and other autofluorescent compounds were detected at 365 nm. Toluene–EtOAc–HCOOH 5:4:1 (solvent system 1) was used for lipophilic compounds and EtOAc-CH_3COOH–MeOH–H_2O 10:2:1:2 (solvent system 2) for moderately hydrophilic compounds.

Reversed-phase HPLC was conducted in the analytical mode on a Zorbax Eclipse XDB-C8 column (5 μm; 4.6×150 mm) using a linear gradient MeCN–H_2O (0.1% TFA) from 5 to 95% MeCN in 40 min and 100% MeCN in 60 min, flow rate 1.0 ml min^{-1}, diode array detection at 200–450 nm, monitoring wavelengths 275 nm. A LiChrospher 100 RP-18 column (10 μm; 250×10 mm) and a linear gradient MeCN-H_2O (0.1% TFA) from 10 to 90% MeCN in 40 min and 100% MeCN in 55 min was used for preparative separation (3.0 ml min^{-1}, UV detection at 275 nm).

4.4. Spectroscopic methods

NMR spectroscopy: ^1H, ^1H–^1H COSY, HMBC, and HMQC spectra of isolated compounds were recorded on a Bruker Avance DRX 500 NMR spectrometer at 500.13 MHz. A microprobe head (2.5 mm) was used for measuring ^{13}C NMR spectra at 125.75 MHz. MeOH-d_4 was used as a solvent and TMS as internal standard. For HPLC-NMR coupling, a HP 1100 chromatographic system and a J&M DAD detector were fitted with a Bruker Avance DRX 500 NMR spectrometer (4 mm inverse detection LC probehead, detection volume 120 μl). HPLC was conducted using a LiChrospher 100 RP-18 column (5 μm; 250×4 mm); linear gradient MeCN-D_2O (0.1% TFA) from 10 to 60% MeCN in 30 min; UV 275 nm; 1.0 ml min^{-1}; stopped-flow mode. ^1H solvent suppression of MeCN and the residual water in the MeCN–D_2O gradients were performed by presaturation, applying standard Bruker pulse sequences. For calibration, the suppressed signal of MeCN was set to δ 2.0. For HPLC-NMR measurements the evaporated fraction was dissolved in 100 μl MeOH-d_4.

Mass spectrometry: Mass spectra were measured using electrospray ionization (ESI). A HP 1100 HPLC (MeCN-H_2O 1:1, C18 column, 5 μm) was coupled to a Micromass Quattro II tandem quadrupole mass spectrometer equipped with an electrospray ionization source. The capillary and cone voltages in ESI mode were 3.3 kV and 18 V, respectively.

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