Characterization of Volatile Constituents from Photomixotrophic Cell Suspension Cultures of *Ruta graveolens*

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Photomixotrophic cell suspension cultures of *Ruta graveolens* were qualitatively and quantitatively analyzed by gaschromatography and mass spectroscopy for volatile compounds. The terpenoid hydrocarbons geijerene and pregeijerene, the C_9-C_{13} methylketones and a series of aliphatic esters, respectively, were found as main constituents. The esters consisted of acetic acid, 2-methylbutyric acid and 3-methylbutyric acid which were esterified with straight chain or branched C_8-C_{11} alcohols. The data are discussed in comparison to previous studies on callus cultures.

Introduction

In the course of our comparative studies on secondary constituents in photomixotrophic and photoautotrophic cell suspension cultures of higher plants [1, 2] green cultures of *Ruta graveolens* have been cultivated. Cell cultures of rue have previously been shown to synthesize coumarins, furanocoumarins, quinolines, and furanoquinolines [3–5] as well as several acridone alkaloids [6–9]. Earlier investigations on volatile compounds from rue callus cultures by Reinhard and associates [10, 11] resulted in the isolation of altogether nine C_9-C_{11} ketones and alcohols which partly occurred in form of their acetates, 2-methylbutyrates and 3-methylbutyrates, respectively. The hydrocarbones geijerene and pregeijerene were also isolated. The influence of cellular differentiation pattern as well as of light conditions on the production of volatiles in *R. graveolens* callus cultures have already been reported [12].

We now describe a detailed analysis of the volatile constituents of photomixotrophic cell suspension cultures of *Ruta graveolens*. These studies are part of a program to compare the different potential for secondary metabolism of heterotrophic and photosynthetically active cell cultures [2].

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, mass spectroscopy; GC, gaschromatography; R, retention time; MW, molecular weight.

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Materials and Methods

Cell cultures

Green callus cultures of *Ruta graveolens*, originally obtained from Dr. E. Reinhard, Tübingen, [3, 10] were kept on Murashige-Skoog medium [13] with 2,4-D and 2% sucrose under continuous white light (9000 lux, Osram L 40 W/25) at 22 ± 1 °C. Dark green sections of this callus material were serially selected until homogeneous, dark green callus cultures were obtained. This latter material was converted into a well growing suspension culture consisting of very small cell aggregates (0.5–1 mm) and a substantial portion of even smaller clusters and single cells. The cell suspension cultures were grown in 200 ml Erlenmeyer flasks with 40 ml MS-medium and 2 g inoculum on a gyrotory shaker, 120 rpm, 22 °C, under the same light conditions as mentioned. Cells were harvested after 14 days in early stationary phase by filtration, washed with fresh medium and excess liquid was removed by suction for 1 min. Cells were stored at − 40 °C until analysis. Chlorophyll content was determined according to [14].

Extraction procedure

Frozen (− 40 °C) cells (250 g) were thawed under methanol (440 ml) and homogenized with a Waring Blendor for 5 min in presence of 100 µg undecanoic acid methylester as internal standard. After filtration of the homogenate, dilution of the methanol to

a concentration of under 35 vol.% and neutralization of the extract with 5% $NaHCO_3$-solution, a liquid-liquid extraction with pentane-methylenechloride (2:1) was carried out. The organic phase was dried over Na_2SO_4 and concentrated according to Drawert *et al.* [15]. The concentrated extract was finally fractionated over a silica gel column with pentane (200 ml, fraction 1), pentane-ether (90:10, 200 ml, fraction 2) and ether (200 ml, fraction 3) as described by Schreier *et al.* [16].

Gaschromatography – mass spectroscopy

Gaschromatography was carried out with a Carlo Erba Fractovap 4130 using a glass capillary Carbowax 20 M column (25 m × 0.25 mm (WGA)). Carrier gas was hydrogen (1.5 ml/min), injector temperature 230 °C, detector temperature 225 °C and temp. program 5 min isotherm to 65 °C, then 2 °C/min up to 190 °C. For identification purposes the retention times of reference compounds were recorded.

Sniffing analyses were conducted with a variable effluent splitter (Siemens, Karlsruhe) using a Scot glass capillary Carbowax 20 M column (40 m × 0.5 mm (SGE)), hydrogen as carrier gas (0.32 bar), injector and detector temperature 200 °C and a temperature program from 65 °C to 190 °C at a rate of 2 °C/min.

For mass spectroscopy a Finnigan model 4021 (quadrupol) was used coupled with a Finnigan gaschromatograph 9610 (glass capillary Carbowax 20 M (Mega) column (25 m × 0.35 mm)). Conditions were: temperature program 5 min isotherm to 65 °C, then 2 °C/min up to 170 °C, helium as carrier gas (38 cm/sec), temperature of injector block and transferline 220 °C, ionisation energy 70 eV, cathode current 25 μA, scan time 0.5 sec for the mass range 33–500.

Mass spectra were recorded with a Nova 4 computer system and stored with a CDC-disc-drive (32 MByte). Spectra were analyzed either by using reference compounds or by comparison with an internal NBS library (33000 spectra); sensitivity was 100 pg methylstearate at *m/e* 298 and a signal/noise-ratio of 3:1 under working conditions. For details see [17].

For quantitative evaluation of spectra the electronic integrator system SP 4000/4020 (Spectra Physics, Darmstadt) was used with undecanoic acid methyl ester as internal standard (100 μg/250 g fresh cells).

Synthesis of reference compounds

Various reference esters of acetic acid, propionic acid, 2-methylbutyric acid and 3-methylbutyric acid were synthesized according to standard procedures [18]. Acetic acid-2-nonyl and -2-undecylester were prepared from acid anhydride with alcohols in pyridin.

The following esters were all synthesized from the acid chloride and the relevant alcohols in pyridin.

From propionic acid were prepared: 2-nonyl and 2-undecylester.

From both 2-methylbutyric acid and 3-methylbutyric acid were prepared: 1-octyl, 2-octyl, 1-nonyl, 2-nonyl, 1-decyl, 2-decyl, 1-undecyl, 2-undecyl and 2-dodecylester.

Results

Cell cultures

Dark green, homogeneous callus cultures of *Ruta graveolens* were turned into photomixotrophic [2] cell suspension which yielded 14 ± 1 g fresh weight from 2 g inoculum in 14 days. At early stationary phase app. 120–130 μg chlorophyll *a* and *b*/per g fresh weight was measured.

Isolation and structural elucidation of volatile compounds

The total methanol extract [15] of 250 g photomixotrophic rue cells was fractionated by silica gel column chromatography into 3 fractions by elution with pentane (fraction 1), pentane/ether (fraction 2) and ether (fraction 3) according to [16].

All fractions were analyzed by gaschromatography-mass-spectroscopy. Compounds were identified by their gaschromatographic retention times and their mass spectra using either authentic reference substances or an internal NBS-library of mass spectra.

Fraction 1 and 2 were also subjected to sniffing analyses.

As described in the Experimental Section several volatile esters had to be synthesized to help in the identification analyses.

Table I. GC-MS analysis of hydrocarbons (fraction 1) obtained from photomixotrophic cell suspension cultures of *Ruta graveolens*. All compounds were identified by a comparison of the measured mass spectra with the spectra of a MS data system.

MS scan number	Compound	Mass spectrum
338	geijerene derivative – 15 *m/e* MW 147	79 (100), 94 (37), 91 (20), 41 (19), 77 (17), 93 (17), 39 (14), 147 (8)
360	geijerene; MW 162	79 (100), 94 (53), 41 (18), 77 (17), 43 (13), 147 (6), 105 (5), 162 (2)
590	mixed spectrum; MW 162	(57 (100), 43 (83), 91 (57), 71 (56), 41 (43),) (105 (31), 85 (30), 119 (6), 147 (6), 162 (6))
725	unsaturated, branched cyclic hydrocarbon; MW 162; Isogeijerene [a]	91 (100), 105 (60), 79 (58), 147 (53), 67 (38), 93 (37), 162 (31), 119 (28), 133 (27)
801	branched, cyclic hydrocarbon; MW 162; Isogeijerene [a]	91 (100), 147 (88), 41 (83), 79 (80), 105 (73), 119 (53), 162 (38), 81 (29)
884	unsaturated branched cyclic hydrocarbon; MW 162; Isogeijerene [a]	79 (100), 91 (87), 105 (65), 77 (43), 93 (36), 119 (28), 133 (24), 147 (37), 162 (3)
973	unsaturated, cyclic hydrocarbon; MW 162; Isogeijerene [a]	91 (100), 147 (99), 105 (69), 41 (48), 162 (37), 67 (33), 77 (32), 119 (28), 133 (17)
1105	pregeijerene; MW 162	79 (100), 94 (30), 41 (17), 77 (18), 91 (17), 105 (14), 147 (6), 162 (8)
2100	acyclic sesquiterpene; Farnesene [a]	93 (100), 41 (97), 69 (64), 55 (60), 119 (52), 79 (34), 91 (30), 107 (26), 135 (5)
2205	acyclic sesquiterpene; Farnesene [a]	41 (100), 93 (76), 69 (67), 55 (67), 91 (37), 77 (27), 67 (13), 53 (22), 119 (15)

[a] Unknown isomeric forms.

Fraction 1 contained a series of hydrocarbons (Table I) which could only partially be identified due to lack of reference compounds. However, geijerene and pregeijerene known from rue callus cultures [11] were unequivocally identified. This fraction requires further investigations to elucidate the various isomeric cyclic hydrocarbons (scans 590, 725, 801, 884, 973) of molecular weight 162 which are considered to be isomerisation products in the geijerene-isogeijerene series. Finally, 2 acyclic sesquiterpene hydrocarbons (scans 2100 and 2205) were found whose spectra are reminiscent of farnesene isomers.

Fraction 2 (Table II) contained as prominent constituents a series of methylketones, long chain aldehydes and alcohols as well as numerous esters of acetic acid, 2-methylbutyric acid and 3-methylbutyric acid, respectively. Thus, 2-nonanone, 2-decanone, 2-undecanone and 2-tridecanone could be identified. Except for the latter ketone these methylketones have also been obtained from rue callus cultures [11].

In addition to small amounts of aldehydes (hexanal, octanal, nonanal and decanal) and the alcohol 1-octanol, fraction 2 contained a complex mixture of esters. Acetic acid was found as the acid moiety in at least 7 esters, 5 of which could unequivocally be identified. These are, acetic acid-1-octylester, acetic acid-1-nonylester, acetic acid-1-decylester, acetic acid-2-nonylester and acetic acid-2-undecylester. The remaining 2 acetic acid esters (scan 1149 and 1610) require reference compounds for further structural elucidation.

Nagel [19] in his studies on volatile constituents of rue callus cultures reported on propionic acid-2-nonylester and propionic acid-2-undecylester. In our analyses these compounds were not detected though authentic samples have been employed. Such discrepancies have to be elucidated in future studies.

Fraction 2 also contained a set of esters of 2-methyl- and 3-methylbutyric acid with straight chain or branched octanol, nonanol, decanol or undecanol, as the alcoholic moiety. Furthermore, various other esters of methylbutyric acid (scans

Table II. GC-MS analysis of ketones, aldehydes, alcohols, and esters (fraction 2) obtained from photomixotrophic cell suspensions of *Ruta graveolens*.

MS scan number	Compound	Identification [a]	Mass spectrum
137	hexanal	MS, R	
315	octanal	MS, R	
532	2-nonanone	MS, R	
545	nonanal	MS, R	
804	acetic acid-2-nonylester	MS, R	
853	acetic acid-1-octylester	MS, R	
902	2-decanone	MS, R	
922	decanal	MS, R	
962	benzaldehyde	MS, R	
1149	acetic acidalkylester	MS	43 (100), 55 (52), 57 (49), 70 (45), 97 (36), 89 (17), 83 (16), 103 (12)
1244	1-octanol	MS, R	
1320	acetic acid-1-nonylester	MS, R	
1388	2-undecanone	MS, R	
1570	2-methylbutyric acid-1-octylester	MS, R	
1610	acetic acidalkylester	MS	
1662	3-methylbutyric acid-1-octylester	MS, R	
1775	acetic acid-2-undecylester	MS, R	
1858	acetic acid-1-decylester	MS, R	
2062	methylpropionic acid- or butyric acidalkylester	MS	71 (100), 43 (68), 41 (20), 55 (14), 89 (15), 85 (12), 115 (13), 70 (11)
2083	ester, alcohol	MS	57 (100), 101 (20), 69 (13), 70 (13), 55 (15), 56 (16), 41 (12), 43 (6)
2116	2-methylbutyric acid-1-nonylester	MS, R	
2186	ester	MS	91 (100), 150 (25), 39 (19), 44 (16), 59 (9), 63, 74, 81, 119, (all < 5)
2218	3-methylbutyric acid-1-nonylester	MS, R	
2516	2-tridecanone	MS, R	
2544	2-methylbutyric acid-2-undecylester	MS, R	
2600	2(3)-methylbutyric acidalkylester	MS	85 (100), 57 (71), 84 (20), 41 (17), 100 (4), 133 (3), 143 (8), 103 (2)
2660	3-methylbutyric acid-2-undecylester	MS, R	
2785	2(3)-methylbutyric acidalkylester	MS	57 (100), 103 (92), 85 (88), 56 (51), 55 (44), 70 (43), 97 (41), 42 (38)
2870	methylpropionic acid alkylester	MS	71 (100), 43 (51), 41 (9), 56 (8), 69 (9), 83, 159, 89, 96, 205, 85 (all < 5)
3421	2(3)-methylbutyric acidphenylester	MS	104 (100), 40 (73), 44 (53), 57 (32), 85 (13), 77, 91, 170, 210 (all < 5)

[a] Means of identification were: MS, comparison of mass spectra with MS data system; R, comparison of GC-retention times with authentic reference compounds.

no. 2600, 2785, 3421) and other, unidentified acids (scans 2083, 2186, 2870) await structural elucidation.

2-methylbutyric acid-2-nonylester and 3-methylbutyric acid-2-nonylester, previously reported by Nagel [19] as callus constituents have not been detected in our analysis. This difference is best explained by assuming that the actually occurring 2-methylbutyric acid-1-nonlyester (scan 2116) and 3-methylbutyric acid-1-nonylester (scan 2218) were erroreously taken for the above mentioned isomers.

Table III. Quantitative composition and sensoric properties (sniffing analysis) of main volatile constituents from photomixotrophic cell suspensions of *Ruta graveolens*.

MS scan number	Compound	Quantity (µg/kg fr. w.)	Sensoric properties
		Fraction 1	
360	geijerene	162	–
801	isogeijerene (isomer)	20	herbaceous, green, rue-typical
1105	pregeijerene	230	herbaceous, rue-typical
		Fraction 2	
1149	acetic acidalkylester	36	–
1320	acetic acid-1-nonylester	138	spicy, sweet
1388	2-undecanone	1575	–
1570	2-methylbutyric acid-1-octylester	147	herbaceous (weak)
1662	3-methylbutyric acid-1-octylester	101	–
1775	acetic acid-2-undecylester	222	–
1858	acetic acid-1-decylester	41	spicy, pungent
2062	ester	14	–
2083	ester	72	–
2116	2-methylbutyric acid-1-nonylester	82	–
2218	3-methylbutyric acid-1-nonylester	70	–
2516/2544	2-tridecanone/2-methylbutyric acid-2-undecylester	190	musty, herbaceous
2660	3-methylbutyric acid-2-undecylester	111	–

Fraction 3 was devoid of any volatile compounds as measured by GC-MS.

In Table III the quantitative composition of the main constituents of the volatile compounds of rue cell suspension cultures are shown. The sensoric properties of some of these compounds as determined by sniffing analyses are also given. 2-undecanone (1.56 mg/kg fresh weight) is by far the most prominent compound. The geijerene-pregeijerene mixture was found to occur to an extent of 412 µg/kg fresh weight. A great variation with regard to quantitative data (14–222 µg/kg fresh weight) was measured for the acetate and methylbutyrate esters.

Discussion

In *Ruta graveolens* plants the hydrocarbons geijerene and pregeijerene are mainly found in the roots whereas the methylketones, the alcohols and the aliphatic esters, respectively, are leaf constituents [20]. Therefore, the mixture of volatiles obtained from *R. graveolens* cell suspension cultures (Tables I and II) contains compounds normally found in both of these two organs. With regard to their biosynthetic potential our photomixotrophic cultures can thus be taken as to combine the biosynthetic properties of both heterotrophic and photoautotrophic organs.

The general composition of our mixture of volatiles fairly well agrees with data obtained with light-grown callus cultures by previous researchers [10, 11]. However, the present analyses as shown in Tables I and II demonstrate that the composition of volatile compounds is much more complex and derived from several, different classes of natural products. The biosynthetic pattern involved will be the object of further studies.

Callus cultures have often been shown to be a rich source of secondary constituents [21] whereas cell suspension cultures were reported to gradually lose this potential unless selection processes for high productivity were carried out [22].

Our qualitative (Tables I and II) and quantitative (Table III) data proof that the cell suspensions now obtained represent a good source of volatiles. It will be of future interest to analyse how this set of volatiles will be effected upon changes of cellular carbon metabolism (photoautotrophic [2]) and nutrient conditions.

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