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Secondary metabolites produced by callus cultures of various *Ruta* species

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Abstract

Callus cultures were established from hypocotyl explants of *R. bracteosa*, *R. chalepensis* and *R. macrophylla*. Calli were maintained for more than three years on MS-medium supplemented with 1 mg l^{-1} of each 2,4-D and kinetin. Acridone and furoquinoline alkaloids and coumarins have been isolated from four week old calli grown on a hormone containing and hormone-free medium. A new chlorinated acridone alkaloid has been detected.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid, MS – medium after Murashige & Skoog [6]

Introduction

Numerous members of the genus *Ruta* (Rutaceae) are rich in secondary metabolites. For example they are able to produce *inter alia* coumarines, flavonoids, lignans, volatile oils, furoquinoline and acridone alkaloids [4, 8].

The first tissue cultures of a *Ruta* species viz. *R. graveolens* were initiated more than 20 years ago [10]. *In vitro* cultures of *Ruta graveolens* have been rather extensively studied. It was found that various cell lines of this particular *Ruta* species accumulate practically the same compounds [9] which have been isolated from the intact plant. Recently suspension cultures of *Ruta* species have been investigated. Under varying culture conditions these cell lines viz. *R. corsica* DC., *R. macrophylla* Moris, *R. chalepensis* L. and *R. bracteosa* DC., were able to produce rutacridone- and hydroxyrutacridone-epoxide [3].

In order to gain more insight in the biochemical capacity of tissue cultures of *Ruta* species other than *R. graveolens* we have investigated three different *Ruta* callus cultures in more detail.

Materials and methods

Culture method

Callus cultures were established from seed samples of *Ruta bracteosa* DC, *Ruta macrophylla* Moris, and *Ruta chalepensis* L. Seeds of *R. bracteosa* and *R. macrophylla* were obtained from the Botanical Garden Kosice, CSFR, and *R. chalepensis* from Siena, Italy.

Seeds were sterilized with concentrated sulfuric acid for 3 min, immediately rinsed with sterile water and put on solidified White's medium [14] for germination. Seedlings were

kept under luminescent lamps (4000 lm m^{-2}) for about three weeks. Pieces of hypocotyls were induced to form callus on solid Murashige & Skoog-medium [6] containing 2,4-dichlorophenoxyacetic acid (1 mg l^{-1}), kinetin (1 mg l^{-1}) and agar (7 g l^{-1}). Furthermore the MS-medium was supplemented during the first five passages with casein hydrolyzate (500 mg l^{-1}) and coconut milk (50 ml l^{-1}). In some cultures the growth hormones were omitted after the tenth passage of cultivation (Table 1). Calli were kept in the dark at 27°C and 70% relative air humidity. Tissues were subcultured every four weeks throughout the last three years. After harvesting four weeks old cells were lyophilized and kept at −20°C.

Isolation of compounds

Lyophilized cell material (10–18 g) was extracted with $CHCl_3$ and subsequently homogenized three times (Ultra-Turrax) in methanol. The combined extracts were concentrated *in vacuo* and then diluted with water. This fraction was exhaustively extracted with $CHCl_3$. The organic phase was dried over Na_2SO_4 and concentrated *in vacuo*. The extract was finally fractionated on a silica gel column (4 × 40 cm) which was equilibrated with $CHCl_3$. The elution was performed with the following solvent systems: a) $CHCl_3$, 1000 ml; b) $CHCl_3$: ethanol (9:1), 500 ml c) $CHCl_3$: ethanol (7:3), 500 ml. From fraction b) rutacridone and the coumarins were separated. From fraction c) the other compounds were isolated. Single compounds were purified to homogeneity by repeated preparative TLC (silica gel Merck PF_{254}) in various solvent systems: I benzene/AcOEt (6:4); II toluene/AcOEt/i-propanol (5:4:1); III toluene/AcOEt/HCOOH (80%ig) (5:4:1); IV toluene/acetone/petrol ether (5:2:3); V $CHCl_3$ (especially for coumarins).

Identification

The compounds were identified by melting point (mp), mass spectrometry (MS) [2] and TLC in various solvent systems and comparison with authentic specimens. On TLC compounds were detected under UV-light (at 254 nm or 365 nm), by methanolic KOH (coumarins), $FeCl_3$- and Dragendorff's reagent (alkaloids).

Rutacridone, mp 160–162°C; rutacridone-epoxide, mp 220-222°C; gravacridonol, mp 152-153°C; gravacridonchlorine, mp 247–250°C, hallacridone, mp 294–296°C; 1-hydroxy-3-methox-N-methyl-acridone mp 159–162°C; arborinine, mp 175–178°C; bergapten, mp 187–190°C; isopiminellin, mp 144–146°C.

Results and discussion

Callus formation of *R-bracteosa*, *R. macrophylla* and *R. chalepensis* was readily induced from hypocotyl explants after one month on MS-medium supplemented with 1 mg l^{-1} of each 2,4-D and kinetin. Callus of *Ruta bracteosa* showed a homogenous undifferentiated growth pattern in the presence of plant hormones. On hormone-free medium partial root formation was observed. Calli of *R. macrophylla* and *R. chalepensis* exhibited a homogenous growth morphological differentiation, but showed a tendency to shoot formation on MS-medium containing 2,4-D and kinetin.

Lyophilized calli of three *Ruta* species cultivated in the presence and without hormones were analyzed for secondary metabolites (Table 1). No differences with regard to the alkaloid and coumarin composition were observed. A number of acridone and furoquinoline alkaloids as well as two coumarins could be isolated from tissue cultures of these particular *Ruta* species. Rutacridone and its derivatives are the main alkaloids in the investigated cell lines. Recently [1, 11] hallacridone was found in tissue cultures of *R. graveolens*. It is now documented that this acetylfuroacridone alkaloid is also synthesized in *R. bracteosa*, *R. macrophylla* and *R. chalepensis*. The same holds true for gravacridonchlorine. Gravacridonol was detected in callus of *R. bracteosa* and *R. macrophylla*. 1-Hydroxy-3-methoxy-N-methyl-acridone has been previously isolated from various Rutaceae species [11] including *Ruta graveolens* tissue cultures [13]. We could show that this simple acridone alkaloid is also present in *R. chalepensis* callus cultures. Arborinine was apparently for the first time detected in *R. bracteosa* tissue cultures.

Table 1. Secondary metabolites produced by callus cultures of various *Ruta* species.

Substance	*R. bracteosa*[1]		*R. macrophylla*		*R. chalepensis*		R_f-values in solvent system	
	a	b	a	b	a	b	II	IV
Rutacridone	1.08[2]	0.6	3.6	1.6	3.1	2.13	0.9	0.81
Gravacridonchlorine	0.09	0.04	0.18	0.15	0.4	+++[3]	0.78	0.52
Rutacridone-epoxide	+	+	–	–	+	+	0.69	0.52
Hydroxyrutacridone-epoxide	++	++	++	++	++	++	0.52	0.16
Hallacridone	++	++	++	++	+	+	0.67	0.48
1-Hydroxy-3-methoxy-N-methylacridone	–[4]	–	–	–	+	+	0.86	0.74
Gravacridonol	++	+++	++	++	–	–	0.54	0.18
A6=unknown	+	+	+	+	+	+	0.54	0.25
Arborinine	+	+	++	++	+	+	0.65	0.47
γ-Fagarine	++	++	++	++	++	++	0.57	0.38
Skimmianine	+	+	+	+	+	+	0.65	0.44
Bergapten	1.25	1.0	0.9	0.5	+++	+++		
Isopimpinellin	2.4	1.76	1.22	0.95	+++	+++		

[1] a: Plant growth substances were omitted since the 10th passage. Harvest of cells for extraction was performed after the 18th passage of cultivation.
b: Cultivation in the presence of growth hormones.
[2] mg g^{-1} dry weight.
[3] Number of crosses indicate amount of substances after TLC.
[4] Not detectable.

The furoquinolines γ-fagarine and skimmianine which we have isolated from our cell material are widely distributed within the Rutaceae. The coumarin fraction of the investigated cell lines contained bergapten and isopimpinellin according to positive and negative MS fragmentation data.

Besides the known secondary metabolites we have isolated by TLC ca. 1 mg of an unknown compound which was designated as A6. The EI MS gave the following signals m/z (rel. int.) 343 (M^+, 30), 341 (M^+, 100), 306 ($M-35^+$, 80), 292 (26), 278 (40), 264 (63), 250 (23), 167 (37). The fragmentation pattern indicates the presence of chlorine in the alkaloid. This compound was further purified by HPLC on Merck Lichrosorb RP 18,7 μm, column 10 × 250 mm, $CH_3OH:H_2O = (6:4)$, flow rate 3 ml min^{-1}. After preparative HPLC the mass spectrum showed the following peaks at m/z 323 (M^+) 306, 294, 292, 278, 265, 241. The ^2H NMR spectrum was fully in accord with that of gravacridonol [7, 12]. Especially informative are the two two-proton signals at 5.3 and 4.3 ppm showing the presence of a hydroxymethylethenyl group. We assume that during evaporation of the polar alkaloid solution after HPLC chlorine was lost and was replaced by OH^- forming gravacridonol. This reaction is favoured by the allylic structure of the side-chain. Therefore, we propose a tentative structure for A6 which is depicted in Fig. 1.

It is feasible that I (A6) is an artefact which might be formed during extraction of *Ruta* cells

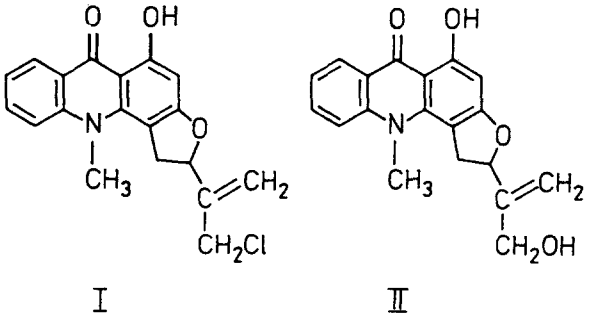

Fig. 1. Tentative structure of alkaloid A6 = I; Gravacridonol = II.

with chloroform. In order to test this hypothesis we have heated under reflux conditions 3 mg gravacridonol dissolved in with HCl acidified $CHCl_3$ for 8 h. After this treatment only gravacridonol but not a trace of I (A6) or another alkaloid could be detected by TLC. Based on this result we assume that I (A6) is a genuine alkaloid which was detected for the first time in *Ruta* species. At present we are engaged in improving the yields of secondary constituents in the investigated *Ruta* callus cultures and to initiate productive cell suspension cultures. Furthermore a total synthesis of I (A6) is under way.

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