Identification of Tropane Alkaloids in Hairy Root Cultures of *Hyoscyamus albus*

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Dedicated to Professor Ernst Reinhard on the occasion of his 65th birthday

GC-MS, Hairy Roots, *Hyoscyamus albus,* Littorine, Tropane Alkaloids

Tropane alkaloids are typical natural products of solanaceous plants. The patterns of these alkaloids from hairy roots of *Hyoscyamus albus* were determined by GC-MS analysis. 18 alkaloidal compounds were detected, six of them only in trace amounts. Some of these alkaloids, namely hygrine, 3α-acetoxytropane, 3β-acetoxytropane and 6-hydroxylittorine as well as the trace compounds N-methylpyrrolidinylhygrine, phenylacetoxytropane and two isomers of feruloyloxytropane were not previously reported to be components of this species. The major compounds formed were found to be hyoscyamine and littorine.

Introduction

The tropane alkaloids hyoscyamine and scopolamine, formed by *Solanaceae* species, are of great pharmaceutical interest. For this reason numerous laboratories have directed a great deal of time and effort with attempts to produce scopolamine and hyoscyamine with plant cell cultures. However the alkaloid levels in disorganized cells are very low. Romeike's excellent work [1] has shown that tropane alkaloids are synthesized in the roots of the intact plant. As the biosynthesis of tropane alkaloids is obviously restricted to an organized state of the tissue, root cultures appear to be a rather more promising system for the production of tropane alkaloids.

So-called hairy roots, obtained by an infection of plants with *Agrobacterium rhizogenes,* are known to synthesize the root-derived natural products of their parent plants. Compared with untransformed root cultures, hairy roots have the advantage of growing fast and without the addition of phytohormones into the culture medium. Moreover, the productivity of hairy roots remains stable for long periods of culture [2].

The alkaloid spectrum of a hairy root clone of *Hyoscyamus albus,* determined by GC-MS, is reported here.

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Materials and Methods

Plant material

Hairy roots of *Hyoscyamus albus* were induced by co-cultivation of wounded leaves of *in vitro* cultivated plantlets with the *Agrobacterium rhizogenes* strain R 1601, which was obtained from Dr. Pythoud, Bern. This supervirulent strain harbours the cosmid pTVK 291 which is responsible for the supervirulent genotype [3]. R 1601 confers a kanamycin resistance gene with the eukaryotic promotor and terminator of the nopaline synthase gene. Kanamycin resistance was used to confirm the transformed state of the roots appearing after cocultivation with the bacteria. A hairy root clone producing high levels of tropane alkaloids was selected.

Culture conditions

The hairy roots were grown in liquid MS medium [4]. The medium was supplemented with 1 g/l casamino acids and 2% sucrose but contained no phytohormones. The roots were cultivated in 100 ml Erlenmeyer flasks containing 25 ml medium on a gyratory shaker at 120 rpm in the dark. The temperature was 25 °C. The roots were subcultured every two weeks.

Alkaloid extraction

0.5 g of fresh roots were homogenized in 25 ml 0.2 M H_2SO_4 and left standing for 2 h. Cell debris was removed by filtration under vacuum. The fil-

trate was adjusted with NaOH to pH 12 and extracted three times with dichloromethane. The organic layer was dried with sodiumsulphate and evaporated. The residue was dissolved in methanol.

Alkaloid analysis

The GC-MS system consisted of a Carlo Erba 5160 GC which was equipped with a 30 m × 0.32 mm fused silica capillary column coated with the methyl silicone stationary phase DB-1 (J & W Scientific, California). Helium was used as carrier gas. Conditions: Injector 250 °C, split 1:20; temperature programme 70–300 °C, 6°/min. The capillary column was directly coupled to the quadrupole mass spectrometer Finnigan MAT 4515. EI-spectra were recorded at 40 eV in combination with the Incos data system. The retention indices were calculated by using co-chromatographed standard hydrocarbons. The identity of the alkaloids was confirmed by comparing the measured data with those of the authentic compounds or with data obtained from the literature [5, 6]. For a separation of hyoscyamine from littorine silylation of the alkaloids was necessary. The probes were evaporated to dryness and treated with MSTFA at 80 °C for 15 min.

Results and Discussion

The time course of growth and alkaloid formation of the *Hyoscyamus albus* hairy root strain examined had been studied previously (data not shown). It had been shown that the alkaloid content reached it's maximum value at the 22nd day of culture. For this reason roots were harvested at the same time. The results discussed below are based upon samples of three subclones of this hairy root strain.

The following alkaloids were identified by GC-MS in extracts of the *Hyoscyamus albus* hairy root cultures:

Hygrine, tropinone, tropine, pseudotropine, 3α-acetoxytropane, 3β-acetoxytropane, cuscohygrine, apoatropine, hyoscyamine, littorine, scopolamine, 6β-hydroxyhyoscyamine. The GLC separation of these alkaloids is illustrated in Fig. 1.

Furthermore in extremely concentrated extracts the following trace compounds could be identified:

Table I. The molecular ions and retention indices of the main alkaloids identified in a hairy root culture of *Hyoscyamus albus*.

Alkaloid	M^+	RI
(1) hygrine	141	1060
(2) tropinone	139	1153
(3) tropine	141	1168
(4) pseudotropine	141	1185
(5) 3α-acetoxytropane	183	1308
(6) 3β-acetoxytropane	183	1315
(7) cuscohygrine	224	1650
(8) apoatropine	271	2020
(9) hyoscyamine	289	2170
(10) littorine	289	2170
(11) scopolamine	303	2293
(12) 6-hydroxylittorine	305	2360

N-methylpyrrolidinylhygrine, 3β-tigloyloxytropane, phenylacetoxytropane, 7β-hydroxyhyoscyamine and two isomers of feruloyloxytropane, probably the *cis*- and *trans*-isomers.

The available information suggests that some of the alkaloids identified, namely hygrine, 3α-acetoxytropane and 3β-acetoxytropane as well as the trace compounds N-methylpyrrolidinylhygrine, phenylacetoxytropane and the two feruloyloxytropane isomers have not been described before for *Hyoscyamus albus* plants or *in vitro* cultures. N-methylpyrrolidinylhygrine was discovered and initially described as a component of *Datura innoxia* roots by Witte *et al.* [6]. The position of the ring fusion could not be deduced from the

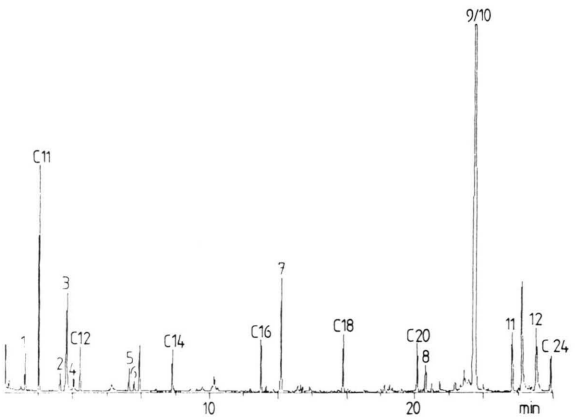

Fig. 1. GLC of a *Hyoscyamus albus* hairy root extract. The peak numbers are identical with the compounds numbered in Table I. The positions of the coinjected standard hydrocarbons are marked by C 11 to C 24.

MS-data but the structure of this alkaloid is suggested to be 4-(1-methyl-2-pyrrolidinyl)hygrine.

The quantitative distribution of hyoscyamine, littorine and scopolamine is interesting. In contrast to other hairy root cultures of the *Solanaceae* this investigation ranks littorine second to hyoscyamine and scopolamine only as a minor alkaloid. Littorine shows values of about one third of the amount of hyoscyamine and the scopolamine content is only one seventh of that of hyoscyamine. This differs from the finding of Hashimoto *et al.* [7], who detected littorine in untransformed root cultures of *Hyoscyamus albus* only in small amounts during the whole period of culture. The quantitative distribution of the three alkaloids differs also when compared with the results of Shimomura and Ishimaru [8]. They detected no littorine in hairy root cultures of *Hyoscyamus albus* but found scopolamine to be a major alkaloid. The *Hyoscyamus albus* strain examined here produced scopolamine only as a minor alkaloid during the culture period of one month. These observations indicate that there are significant differences in the quantitative and possibly also qualitative alkaloid composition of different hairy root strains of *Hyoscyamus albus*. Further investigations will show whether these differences are due to the different genotype of the plant material or are based upon genetic or physiological changes due to the transformation by the *Agrobacterium* strains used.

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