Umbelliferone in Needles of *Picea abies* *

Hermann Esterbauer, Elisabeth Schwarzl, and Dieter Grill

Institute of Biochemistry and Institute of Plant Physiology University of Graz, Halbärthgasse 5/I, A-8010 Graz, Austria

Z. Naturforsch. **35 c,** 682–684 (1980); received May 12, 1980

Umbelliferone, Quantitative Estimation, *Picea abies*, Genetic Families

A compound showing blue fluorescence under UV-light was isolated from spruce needles and identified as umbelliferone. Structure analysis was based on UV and mass spectroscopy as well as chromatographic comparison with authentic material. Quantitative evaluation by direct measurement of the fluorescence of the umbelliferone spot separated by TLC revealed that the umbelliferone content shows great variation within different individual trees ranging from 0.42–42 µg/g fresh weight. Only minute amounts are present as free umbelliferone while the abundant part is present as β-glucoside.

Introduction

During the study of phenolic constituents [1–4] of needles from *Picea abies* (L.) Karst. we often observed on the thin layer chromatograms a substance with bright blue fluorescence in the UV-light. The R_f value in different solvents and the fluorescent color indicated that the substance might be umbelliferone (7-hydroxycoumarine). Umbelliferone is known as common constituent of Apiaceae and some other not related families but not of coniferae [5, 6]. In order to definitely prove the presence of umbelliferone in spruce needles we have isolated a small amount of the fluorescent substance and ascertained its structure by spectroscopic methods. In addition, a procedure was developed which allows the quantitative estimation of nanograms of umbelliferone in plant material.

Materials and Methods

Needles of *Picea abies* were collected from about 10 years old trees of a forest near Graz. Authentic umbelliferone was from Roth (Karlsruhe), β-glucosidase was purchased from Sigma (Saint Louis). All reagents used were per analysis from Merck (Darmstadt). The mass spectra were recorded with a AEI, MS 20 instrument, ionisation energy 70 eV, temperature of ion source 120 °C. The UV spectra were recorded with a Zeiss PMQII. Direct measurements of fluorescence on the TLC plates were performed by scanning the plates with a Zeiss Chromatogram-Spectrometer equipped with a fluorescent attachment ZFM4.

Isolation of umbelliferone

A pooled sample (100 g) of previous year needles was homogenized (Sorvall omnimixer, full speed) with H_2O (2 × 500 ml). The combined filtered extracts were adjusted to pH 1 with HCl conc., boiled for 30 min and extracted with Et_2O. The ether extract was dried with anhydrous calcium chloride and then separated by repeated column chromatography (silica gel, toluene/$CHCl_3$/acetone 40:25:25 and benzene/Et_2O 1:1) followed by TLC (silica gel, benzene/Et_2O 1:1). The zone with bright blue fluorescence in the UV-light corresponding in the R_f value to umbelliferone was scrapped off, eluted with methanol and dried. After recrystallization from water about 1 mg of a white, solid substance was obtained.

Quantitative estimation of total umbelliferone

Needles (1 g) were homogenized (Ultra Turrax, full speed) for 3 min with 10 ml distilled water. The suspension was centrifuged and the sediment was homogenized again with 10 ml water. The combined clear supernatants were treated with about 50 mg β-glucosidase (5 units/mg) for 12 h at room temperature. The precipitate formed was centrifuged and discarded. The supernatant was extracted 4 times with 20 ml ethyl acetate. The pooled extract was evaporated and the dry residue was redissolved in 0.1 ml ethyl acetate. 2 and 5 µl fractions of this solution as well as umbelliferone standards (10–

* We dedicate this work to Prof. Dr. G. Zigeuner on the occasion of his sixtieth birthday.

Reprint requests to Prof. Dr. H. Esterbauer.

0341-0382/80/0900-0682 $ 01.00/0

200 ng) were applied to precoated silica gel glass plates 20 × 20 cm (Merck) and developed with toluene/$CHCl_3$/acetone/acetic acid 40:25:35:2. The TLC-plates were then quantitatively evaluated by direct scanning of the fluorescence of the umbelliferone spots with the chromatogramspectrometer, using excitation 330 nm and emission 395 nm. The amount of umbelliferone in the experimental sample was then determined in the usual way from the calibration curve established with the peak areas of the standards.

Thin layer chromatography

The following solvents were used for chromatography of authentic and isolated umbelliferone resp.

A: $CHCl_3$/methanol 97:3, R_f 0.74,
B: benzene/acetone 90:10, R_f 0.39,
C: benzene/ether 50:50, R_f 0.50,
D: benzene/chloroform 50:50, R_f 0.04,
E: toluene, ethylformiate/formic acid 50:40:10, R_f 0.59,
F: toluene/chloroform/acetone 40:25:35, R_f 0.67,
G: toluene/chloroform/acetone/acetic acid 40:25:35:2, R_f 0.67.

Results and Discussion

When an ether or ethylacetate extract of an aqueous spruce needle homogenate was separated by TLC (solvent C) the resulting chromatogram showed besides a number of other fluorescent spots one of R_f 0.50 with a very characteristic bright blue fluorescence. The fluorescent intensity of this spot considerably increased when the needle homogenate was treated with HCl or β-glucosidase before extraction with the organic solvent. The color of the fluorescence and the R_f value pointed to umbelliferone, a coumarine derivative (7-hydroxycoumarine) not known as common constituent in coniferae. To prove this preliminary assumption we have isolated the fluorescent substance for additional structure analysis.

For the isolation of the compound we started from a HCl hydrolyzed spruce needle extract and purified the compound by repeated column chromatography and TLC separations and finally by recrystallization. From 100 g needles (fresh weight) we obtained about 1 mg of the chromatographically pure substance.

Evidence for the structure of the isolated compound was based on the mass spectrum, the UV spectrum in neutral (EtOH) and alkaline (EtOH/KOH) solution and TLC separation in seven different solvents. The mass spectrum is shown in Fig. 1. The compound exhibited the expected molecular ion peak ($M^+ = 162$) which is also the base peak of the spectrum. The main fragmentation process is the loss of one molecule of carbon monoxide leading to the peak $m/e = 134$ followed by a loss of a second molecule of CO or a formyl radical (CHO) giving the peaks at $m/e = 106$ and $m/e = 105$ resp. The mass spectrum was in all details identical with the spectrum of authentic umbelliferone. The identification of the isolated substance was further confirmed by the UV spectra. The substance showed in EtOH an intense maximum at 326 nm which shifted to 377 nm in EtOH/0.1 N KOH. The position of the absorption bands and the shape of the spectra were in complete agreement with the reference umbelliferone. To provide further proof for the structure the isolated compound was chromatographed together with reference umbelliferone on thin layer plates in seven different solvents (see experimental part). In each solvent agreement of the R_f value was established.

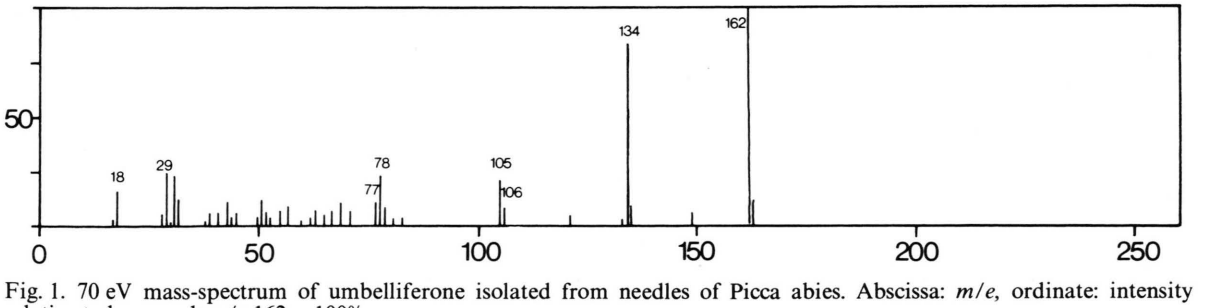

Fig. 1. 70 eV mass-spectrum of umbelliferone isolated from needles of Picca abies. Abscissa: m/e, ordinate: intensity relative to base peak m/e 162 = 100%.

Table I. Total umbelliferone content in needles of *Picea abies*. 1st year needles (December 1976) of trees from different stands (No. 1–10) were homogenized, hydrolyzed with β-glucosidase and analyzed as described in the experimental part.

Tree No.	µg/g fresh weight
1	0.42
2	0.55
3	0.61
4	0.71
5	1.21
6	1.44
7	3.55
8	6.80
9	8.80
10	42

To measure the amount of umbelliferone present in spruce needles the extract and standards were separated by TLC and quantitatively evaluated by direct measurements of the fluorescence of the spots. In the range of 5–200 ng a linear relation exists between the fluorescence intensity of the spots (measured as peak area) and the amount of umbelliferone applied to the plate.

Free umbelliferone was present in most individuals only in very low concentration ranging from not detectable amounts to 2 ng/1 g fresh weight. When the needle homogenate was treated during 12 h with β-glucosidase a considerable increase of umbelliferone was observed. Hence it follows that the abundant part of umbelliferone is present not in the free form but as β-glucoside (umbelliferone-7-glucoside = Skimmin). Table I shows total umbelliferone *i.e.* free + β-glucoside in the needles of 10 different trees. It is evident that umbelliferone exhibits a great variability covering the range of two orders of magnitude. The reasons for this individual variation are difficult to be explained. It can be excluded that low umbelliferone values are due to a limited availability of *p*-cumaric acid which is the precursor for its biosynthesis since *p*-cumaric acid is always present in sufficient excess ranging from 170–1500 µg/1 g fresh weight [4]. Environmental and ecological reasons can also be rooled out because high disagreement of the umbelliferone content was also observed in trees growing in close neighbourhood. It seems most likely that the umbelliferone level is genetically controlled, and that trees possessing similar umbelliferone contents belong to genetically related families. On the other hand, the umbelliferone content was rather constant within one individual tree. Measurement conducted over a nine month period (October 1976 to July 1977) with a test-tree revealed an average of 3.26 ± 0.29 µg/g fresh weight with no indications for significant maxima or minima. Moreover, 1st, 2nd and 3rd year needles had similar umbelliferone contents with a variation not greater than ± 20%. In the needles of the test-tree values in June for instance were: 1.70, 3.24 and 2.99 µg/1 g fresh weight. Umbelliferone thus is a constituent with great variability within different individuals and low variability within one individual.

Only little is known on the physiological role of umbelliferone. It is possibly involved in growth regulation [5] and in the defense mechanism against microbial infection [7]. It is likely that umbelliferone is under genetic control and that trees with similar contents belong to genetically related varieties. Böritz [8] has made an attempt to classify related varieties of *Picea abies* based on the fluorescence intensities of various spots on the paper chromatogram of the needle press juice. He points to a substance with a bright blue fluorescent which is present in very different amounts in needles from different trees. Based on our results we assume that this substance was umbelliferone and that its quantitative measurement, as described in this paper, could give a valuable parameter for genetic family analysis of *Picea abies* in natural forests.

Acknowledgement

The work was supported by a grant from the Fonds zur Förderung der wissenschaftlichen Forschung, Wien.

[1] H. Esterbauer, D. Grill, and G. Beck, Anal. Chem. **46,** 789–791 (1974).
[2] H. Esterbauer, D. Grill, and G. Beck, Phyton **17,** 87–99 (1975).
[3] W. Lindner and D. Grill, Phyton **18,** 137–144 (1978).
[4] E. Schwarzl, Thesis, University of Graz 1978.
[5] P. Jankay and W. H. Muller, Amer. J. Bot. **63,** 126–132 (1976).
[6] G. J. Niemann, Acta Bot. Neerl. **28,** 73–88 (1979).
[7] J. B. Harborne, Biochemistry of Phenolic Compounds, Academic Press, London-New York 1964.
[8] S. Börtitz, Flora **153,** 320–325 (1963).