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A HYDROXYBUTENOLIDE FROM *SPHAGNUM* SPECIES

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Key Word Index—*Sphagnum magellanicum*, Sphagnaceae; bryophyta; sphagnum acid; degradation; peroxidase; isoenzymes; hydroxybutenolide, cell wall.

Abstract—One of the main products formed by peroxidative degradation of sphagnum acid *in vitro* is identified as (−)2,5-dihydro-5-hydroxy-4-[4′-hydroxyphenyl]-furan-2-one, abbreviated as hydroxybutenolide. It seems to be the first report of the isolation of this compound. Peroxidase isozymes isolated from *Sphagnum magellanicum* have specific preferences in degrading sphagnum acid. Hydroxybutenolide is a naturally occurring component in *Sphagnum* spp. Occurring mainly bound to the cell wall. In contrast to sphagnum acid, hydroxybutenolide is not excreted into the surrounding medium by living *Sphagnum* plantlets.

INTRODUCTION

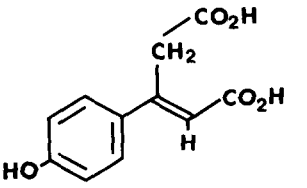
The main phenolic compound masking cellulose in the cell walls of *Sphagnum* has been identified as *p*-hydroxy-*β*-[carboxymethyl]-cinnamic acid, or sphagnum acid [1]. All *Sphagnum* species analysed contain this cinnamic acid derivative in various amounts depending on species, seasons, and plant parts investigated. The sphagnum acid content diminishes in the stem segments of the plants whereas high peroxidase activities are measurable there [2, 3].

Sphagnum acid (**1**) is degraded by peroxidases *in vitro* [4]. One of the main degradation products is a substance preliminarily called compound 'X'. Using high resolution mass spectrometry the molecular formula of this component was determined to be $C_{10}H_8O_4$ [5]. Compound 'X' has been detected as a naturally occurring substance in ethanol extracts of 30 *Sphagnum* species investigated [2]. In the present paper we have elucidated the structure of 'X' and analyse in more detail the degradation of this cinnamic acid derivative by peroxidase isoenzymes isolated from *Sphagnum magellanicum*.

RESULTS AND DISCUSSIONS

Hydroxybutenolide, an in vitro *degradation product of sphagnum acid*

Sphagnum acid (**1**), synthesized as described in ref. [6], was degraded *in vitro* by peroxidase isolated from capitula of *Sphagnum magellanicum*. One of the main degradation products separated by column chromatography was crystallized The molecular formula $C_{10}H_8O_4$ was verified by mass spectrometry. By means of NMR-spectroscopy and α_D, the structure was shown to be (−)2,5-di-hydro-5-hydroxy-4-[4′-hydroxyphenyl]-furan-2-one, or hydroxybutenolide (**2**).



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Hydroxybutenolide is stable in aqueous soln in a pH-range from 3 to 11, and at temperatures up to 50°. In contrast to previous reports [3, 5] hydroxybutenolide, in sufficient concentrations, forms coloured complexes with Pauly's or Millon's reagent.

Sphagnum-peroxidase (SPOD)-isoenzymes and the in vitro *degradation of sphagnum acid*

Recently, in *Sphagnum magellanicum*, 13 basic and five acidic SPOD-isozymes were revealed by polyacrylamide gel electrophoresis [7]. Of the 13 basic isozymes, 10 are buffer-soluble. One of the basic isozymes (No. 6) is located exclusively in the cell walls, two (No. 8 & No. 10) both in the cell wall and the cytoplasm.

The isozymes listed in Table 1 were studied in respect to sphagnum acid degradation. Isoenzyme No. 10 has a preference for degrading sphagnum acid. Among the degradation products detected were 2.9 nmol hydroxybutenolide and 6 nmol *p*-hydroxybenzoic acid. In comparison to isoenzyme No. 10, the degradation of sphagnum acid by the isoenzyme No. 11 is small (1.6%), but by contrast the relative amounts of hydroxybutenolide and *p*-hydroxybenzoic acid formed are the highest. Of the degraded sphagnum acid, 25% is transformed to *p*-hydroxybenzoic acid and 3.8% to hydroxybutenolide. The isoenzyme No. 6, exclusively bound to the cell wall, could not be prepared in sufficient amounts for degradation studies.

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Table 1 Degradation of sphagnum acid by several SPOD isoenzymes and formation of hydroxybutenolide and *p*-hydroxybenzoic acid

Isoenzyme isolated	No 4	No 5	No 8	No. 9	No 10	No 11
nmol sphagnum acid (**1**) degraded/μg protein	197	82	181	76	507	8
nmol hydroxybutenolide (**2**) formed/μg protein	2 2	0.7	1 6	--	2 9	0 3
nmol *p*-hydroxybenzoic acid formed/μg protein	32	10	17	5	6	2

Hydroxybutenolide a natural compound in living Sphagnum

Living *Sphagnum* plantlets were homogenized in phosphate buffer. A 20 000 *g* supernatant and a 80 μm cell wall particle fraction were analysed for sphagnum acid and hydroxybutenolide. In the buffer fraction 113 nmol sphagnum acid and 40 nmol hydroxybutenolide were detected per 100 mg dry wt. The amounts of both components extractable from the 80 μm cell wall fraction with alkaline ethanol were significantly higher, 468 nmol sphagnum acid and 266 nmol hydroxybutenolide respectively. Therefore 81% of the sphagnum acid and 87% of the hydroxybutenolide are located in the cell walls of the plantlets. These data fit in with the POD localization ultracytochemically detected in differentiated *Sphagnum magellanicum* leaves, recently presented [5]

To study the binding of hydroxybutenolide and sphagnum acid to the cell wall in more detail, we analysed the time course of extraction by ethanol (24 hr-fractions). The greatest amounts corresponding to 100 mg dry wt are gained in the first three fractions, 31 μg sphagnum acid and 16 μg hydroxybutenolide, but even in the fraction 17 both compounds are still detectable. Thus within 408 hr 52 μg sphagnum acid and 44 μg hydroxybutenolide are obtained. The yield of a subsequent extraction with alkaline ethanol for 10 hr (5 × 2 hr-fractions) results in 50 μg sphagnum acid and 14 μg hydroxybutenolide. An additional extraction (5 × 2 hr) with acidic ethanol releases further amounts of both compounds. 32 μg sphagnum acid and 12 μg hydroxybutenolide per 100 mg dry weight In all, 134 μg sphagnum acid and 70 μg hydroxybutenolide are extractable by the sequence of extraction steps mentioned above, whereby both substances are present in the molar ratio of nearly 5 3. These results indicate that both compounds are closely bound to cell walls The fact that increased amounts are extractable by subsequent weak alkaline or acidic conditions indicates esterlike bonds

The excretion of sphagnum acid and hydroxybutenolide into the surrounding medium by living Sphagnum *plants*

After cultivating *Sphagnum* in a fermenter under axenic conditions as described in ref. [8], sphagnum acid was measured in the nutrient solution in a concentration range of 0 5–5 μM which was dependent on nutrient conditions An analysis of bog water revealed sphagnum acid in a range of 0.1–1 μM. In contrast to sphagnum acid, hydroxybutenolide could not be detected in an external media Unsaturated lactones, especially the 2(5H)-furanone unit occur widely in nature [9] Many of these compounds show biological activity [10, 11]. The relevance of the natural compound described in this paper and sphagnum acid in the metabolism of *Sphagnum* is unknown A study of the relationship of these compounds to cell wall structure and of the possible ecological role of the excreted sphagnum acid will require new experimental approaches

EXPERIMENTAL

Plant material Sphagnum magellanicum plants were collected in the Kaltenhofer Moor in Schleswig-Holstein

Extraction of phenolic compounds The extraction procedure was as described by ref. [12]. The pre-extracted moss powder was incubated with 100 ml alkaline EtOH (11 22 g KOH/100 ml EtOH) and in addition with acidic EtOH (36 5% HCl–EtOH, 1 5) at 20°

Hydroxybutenolide Sphagnum acid was degraded as described in reference [5], hydroxybutenolide was isolated by CC with cellulose, eluting with MeOH–H_2O (7 93) by an ascending procedure Colourless needles (H_2O), mp 228° (uncorr.), $[\alpha]_D^{20}$ −3 5° (MeOH, *c* 0 5537), ^{1}H NMR (360 13 MHz, DMSO-D_6) δ 10.28 (1H, *s*, OH), 7 96 (1H, *d*, J = 9 Hz, OH), 7 72 (2H, *m*, H-2′ and H-6′), 6 94 (2H, *m*, H-3′ and H-5′), 6 58 (1H, *d*, J = 9 Hz, H-5) 6 57 (1H, *s*, H-3) ^{13}C NMR (90 56 MHz, DMSO-d_6): δ 171 38 (*s*, C-2), 163 57 (*s*, C-4), 160 56 (*s*, C-4′), 130 37 (*d*, C-2′ and C-6′), 120 81 (*s*, C-1′), 115 87 (*d* C-3′ and C-5′) 111 19 (*d*, C-3), 97 97 (*d*, C-5) EIMS (70 eV), *m/z* 192 $[M]^+$

Chromatographic detection by HPLC A KONTRON high-performance liquid chromatograph, 5 μm ODS II column, 4 6 × 250 mm, 20 μl sample loop were used for the analysis of the phenolic compounds The solvent system consists of HCO_2H–H_2O (1 19, v/v) (A) and MeOH (B) A gradient consisting of 7% B in A, held for 5 min and increased linearly to 80% B over the next 40 min, was used (flow-rate 1 ml/min) The compounds were detected at 280 nm.

Enzyme preparation Ca 50 g of green *Sphagnum magellanicum* plants were homogenized in Pi buffer (0 1 M, pH 7.5) at 4°, using a cell homogenizer MSK (Braun Melsungen) by means of glass-beads, filtrated and centrifuged for 20 min at 20 000 g, to separate cell wall fragments and organelles The supernatant was brought to a vol of 15 ml by Amicon (PM-10 filter) and contained the enzyme used for prep gel electrophoresis and degradation assay

Electrophoretic assays The basic isoperoxidases were separated electrophoretically using a 7%–pH 8.9 polyacrylamide gel system. The composition of the stacking gel was 3 3% (w/v) acrylamide, 0 09% (w/v) bisacrylamide, 58 75 mM Tris–Pi, pH 6 9, 0 057% TEMED and 0 07% (w/v) ammonium persulphate

The running gel was 8.5% (w/v) acrylamide, 0.23% (w/v) bisacrylamide, 755 mM Tris–HCl, pH 8.9, 0.05% TEMED and 0.14% (w/v) ammonium persulphate The electrode buffer used was 38.3 mM glycine and 4.95 mM Tris with the pH adjusted to 8.3 with HCl. Electrophoresis was carried out in slab gels at 15 mA (stacking gel) and 40 mA (running gel) constant current at 4°. The bands were stained by a mixture of 75 ml Pi buffer (0.1 M, pH 5.0), 25 ml soln containing 0 03% *o*-dianisidine in EtOH–H_2O (1:5) or 0 4 ml guaiacol and 0.5 ml 35% H_2O_2. The separated isoenzymes of 6 electrophoretic runs were isolated by electro-elution at 100 V for 8 hr and used for degradation assay and protein determination according to ref. [13].

Degradation assay For degradation of sphagnum acid by SPOD isoenzymes the following incubation mixture was used: 0 4 mmol sphagnum acid in 80 ml 0.1 M acetate buffer, pH 5.0. 40 ml SPOD, 32 nkat/ml, 40 ml 1 19 μmol H_2O_2, adjusted to pH 5.0. The reaction was stopped by liquid N_2 after 5 min.

Isolation of cell wall particles. 25 g *Sphagnum* capitula fresh wt were homogenized in Pi buffer (0.1 M, pH 5.5) using an Ultra-turrax (2 × 2 min) and a Braun cell homogenizer. Firstly the homogenate was filtered through a 230 μm gauze filter, homogenized again and passed through a 80 μm gauze filter. The resulting cell wall suspension with 80 μm or less in size was centrifuged (250 *g*, 3 min), the pellet resuspended and washed × 5 with Pi buffer (5 × 40 ml).

REFERENCES

1. Tutschek, R., Rudolph, H., Wagner, P. H. and Kreher, R. (1973) *Biochem. Physiol. Pflanz* **164**, 461.
2. Rudolph, H. and Samland, J. (1985) *Phytochemistry* **4**, 745.
3. Tutschek, R. (1979) *Z. Pflanzenphysiol* **94**, 317.
4. Tutschek, R. (1979) *Phytochemistry* **18**, 1437.
5. Wächter, D., Rudolph, H. and Völker, H. (1987) *J. Plant Physiol.* **126**, 449.
6. Wächter, D and Rudolph, H (1984) *Z. Naturforsch.* **39c**, 311
7. Matlok, J. and Rudolph, H. (1988), PSE-Symposium: Chemistry and Chemical Taxonomy of Bryophytes, Saarbrücken, poster presentation
8. Rudolph, H., Kirchhoff, M. and Gliesmann, S. (1988) in *Methods in Bryology. Proc. Bryol. Meth. Workshop, Mainz* (Glime, J., ed.), p 25. Hattori Bot. Lab. Nichinan
9. Rao, Y. S. (1975) *Chem. Rev.* **76**, 625.
10. Haynes, I. J (1948) *Q. Rev. Chem. Soc.* **2**, 46.
11. Namiki, T., Baba, Y., Suzuki, Y., Nishikawa, M., Sawada, K., Itoh, Y., Oku, T., Kitaura, Y and Hashimoto, M. (1988) *Chem. Pharm. Bull.* **36**, 1404.
12. Wilschke, J and Rudolph, H. (1988) in *Methods in Bryology. Proc Bryol. Meth. Workshop, Mainz* (Glime, J , ed.), p. 165. Hattori Bot Lab., Nichinan.
13. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.