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COMPARTMENTALIZATION OF PHENOLIC CONSTITUENTS IN *SPHAGNUM*

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Key Word Index—*Sphagnum* species; Sphagnaceae; Bryophyta; compartmentalization; phenolics; *cis*-/*trans*-sphagnum acid; sphagnum acid ethyl ester; hydroxybutenolide; *p*-hydroxyacetophenone.

Abstract—[*E*]-4-ethoxycarbonyl-3-(4′-hydroxyphenyl)-but-2-en-1-carboxylic acid (*trans*-sphagnum acid ethyl ester) and *p*-hydroxyacetophenone were isolated from ethanolic extracts of *Sphagnum magellanicum* cell walls and [*Z*]-3-(4′-hydroxyphenyl)-pent-2-en-1,5-dicarboxylic acid (*cis*-sphagnum acid) from irradiated *trans*-sphagnum acid solutions. The structures of these compounds were elucidated by means of NMR spectroscopy. Compartmentalization studies revealed that the main part of detectable sphagnum acid is buffer-soluble, as well as in *Sphagnum* collected from natural sites and in *Sphagnum* cultivated in bioreactors. Cultivating *Sphagnum* species in bioreactors under axenic conditions leads to an enhancement of the buffer-soluble part of all analysed phenolics with a concomitant decrease of their cell wall-bound amount. Sphagnum acid, sphagnum acid ethyl ester, hydroxybutenolide, *p*-hydroxybenzoic acid, *p*-coumaric acid and *t*-cinnamic acid are also excreted to a remarkable extent into the effluent culture media of *S. fallax* and *S. cuspidatum*. Specific compartments for the storage of endogenous free water-soluble phenolics are postulated.

INTRODUCTION

Apart from the common cinnamic and benzoic acid derivatives two phenolics have been described as unique for *Sphagnum*, sphagnum acid (*p*-hydroxy-*β*-[carboxymethyl]-cinnamic acid) [1–3] and hydroxybutenolide {(-2, 5)-dihydro-5-hydroxy-4-[4′-hydroxyphenyl]furan-2-one} [4]. All *Sphagnum* species analysed contain both compounds, the determinable amount depending on species, season and plant part investigated [5]. The technique to cultivate *Sphagna* axenically in continuous feed bioreactors initiated studies on the metabolism of sphagnum acid [6]. Tracer experiments gave evidence for the formation of the carboxymethyl side-chain of sphagnum acid from acetate or a derived product transferred on a phenylpropane unit [7]. On the other hand, peroxidases degrade sphagnum acid; hydroxybutenolide and *p*-hydroxybenzoic acid are two of the isolated degradation products [4, 8].

As a rule, freeze-dried moss material was used for all studies, so sphagnum acid, hydroxybutenolide, as well as *p*-hydroxybenzoic acid, have been described as being predominantly localized in the *Sphagnum* cell wall. The incorporation of these phenolics into cell walls occurs at later stages in differentiation, masking the histochemical detection of cellulose in the *Sphagnum* cell wall concomitant with an increased resistance of the cell walls against enzymatic degradation [9, 10]. This led us to favouring the hypothesis that these phenolics might serve a function similar to that of lignin in imparting apparent resistance to *Sphagnum*, although the lack of typical *β-O*-4- and phenylcoumaran ^{13}C NMR signals excludes the existence of lignin in *Sphagnum* cell walls [10].

In the recent past, sphagnum acid, as well as hydroxybutenolide, were detected also as endogenous free water-soluble components in buffer homogenates prepared from living *Sphagnum* plantlets. Moreover, analysis of bogwater revealed that sphagnum acid is also excreted into the external medium; concentrations of 10^{-6}–10^{-7} M have been measured [4]. Accordingly, there is the necessity for studies on the metabolism of phenolics in *Sphagnum* species in order to determine separately the amount of phenolics excreted into the external medium, the amount deposited in the cell wall and the content of endogenous free water-soluble phenolics. We present such compartmentalization studies for *Sphagnum* collected from natural sites and *Sphagnum* species cultivated in bioreactors. Furthermore, we emphasize some important methodical aspects in the determination of phenolics in *Sphagnum* and describe in this context the isolation of sphagnum acid ethyl ester, *p*-hydroxyacetophenone and *cis*-sphagnum acid.

RESULTS AND DISCUSSION

Methodical aspects

According to Tutschek [8], sphagnum acid (**1**) is quantitatively extractable from lyophilized *Sphagnum*

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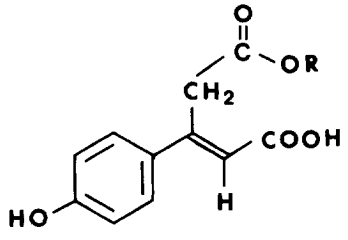
material, ground in a micro-dismembrator, within 4 hr with hot 96% ethanol. Application of the more sensitive HPLC technique, however, revealed that such an extraction procedure is insufficient. A standard extraction procedure with alkaline ethanol was proposed because of the higher amounts of detectable sphagnum acid under these conditions [11].

However, free sphagnum acid is partially esterified to [*E*]-4-ethoxycarbonyl-3-(4′-hydroxyphenyl)-but-2-en-1-carboxylic acid, for short, *trans*-sphagnum acid ethyl ester (**2**), during extraction with hot 96% ethanol, especially under acidic conditions. Acidification depends on cation content and cation-exchange capacity of the moss material used. We isolated the ester from ethanolic extracts of *S. magellanicum* and elucidated its structure by NMR spectroscopy. The ^{1}H and ^{13}C NMR spectra verified the presence of an [*E*]-sphagnum acid moiety in which all proton and carbon chemical shifts were in good agreement with those reported [12]. The remaining signals, ^{13}C: δ14.4 (*q*), 60.9 (*t*), ^{1}H: δ1.15 (*t*) and 4.06 (*q*), belonged to an ethoxycarbonylic group positioned at C-4. In the coupled ^{13}C NMR spectrum, the carboxylic C-atom showed a multiplet structure due to the coupling of both the H-4 methylene and the methyleneoxy protons, while the C-1 signal was a doublet.

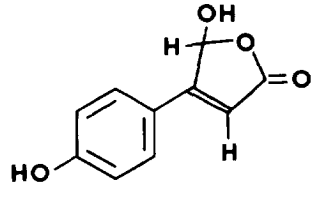
In experiments with synthetic sphagnum acid [13], the acid is 36% esterified within 24 hr in boiling 96% ethanol–N hydrogen chloride (100:0.02). The ester is saponified rapidly in hot alkaline ethanol and, therefore, not detectable in alkaline cell wall extracts. The total amounts of free sphagnum acid and sphagnum acid ethyl ester detectable in ethanolic extracts are the same as the amount of free sphagnum acid in alkaline ethanolic extracts. Although extraction with alkaline ethanol seems to be a suitable method to prevent ester formation, extraction with hot 96% ethanol has to be preferred, since under alkaline conditions, hydroxybutenolide (**3**), another characteristic constituent of all *Sphagnum* species investigated, is degraded by 50% after 1 hr in boiling ethanol–0.1 N sodium hydroxide (w/v). Thus, for ethanolic extracts, the amounts of sphagnum acid and sphagnum acid ethyl ester have to be summed up (Table 1), since it is not certain whether the ethyl ester is only formed during the extraction procedure, or, is at least partially, a native component of the cell walls. Sphagnum acid ester was detectable in buffer homogenates of the *Sphagnum* species investigated and was also excreted under specific conditions into the surrounding medium (Table 2); no ethanol was added during these experiments, indicating that it was a native compound of *Sphagnum*.

A further native constituent isolated was *p*-hydroxyacetophenone (**4**), which is very common in higher plants, often isolated as its glucoside, picein. The fact that this compound is not referred to as an important native constituent of *Sphagnum* species may be due to the detection techniques used. *p*-Hydroxyacetophenone shows very similar chromatographic behaviour to sphagnum acid by HPLC and TLC. By using an improved HPLC gradient we succeeded in separating both components and in isolating the *p*-hydroxyketone. The structure was elucidated by means of its known spectroscopic data which were compared with those from an authentic sample. A reinvestigation of the peroxidative degradation of sphagnum acid [4, 14], resulted in the detection of *p*-hydroxyacetophenone as a further degradation product, apart from the already described hydroxybutenolide and *p*-hydroxybenzoic acid.

The ability to cultivate *Sphagnum* species in bioreactors presents the possibility of analysing the excretion of metabolic products into the surrounding culture medium. On the other hand, precursors can be exogenously supplied. In such studies on the excretion and uptake of sphagnum acid, we found that a photon flux density of 105 μmol m^{-2} s^{-1} offered during the light period of 14 hr [6] induces a 46% conversion of *trans*-sphagnum acid to its *cis*-isomer.



1 R = H
2 R = CH_2CH_3



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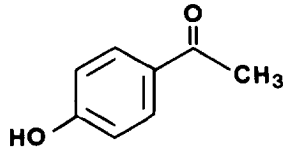

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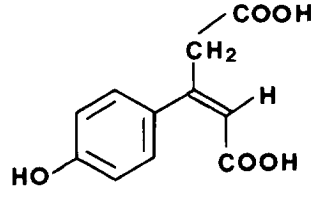

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Table 1. Buffer-soluble and cell wall-bound phenolic constituents of *Sphagnum*

Phenolic compound	*S. magellanicum**	*S. fallax**	*S. fallax*†	*S. cuspidatum*†
Sphagnum acid				
Total amount				
[μmol g^{-1} dry wt]	2.3	2.9	2.0	2.2
Distribution in %				
I	58	61	78	78
II	27	24	15	15
III	15	15	7	7
p-Hydroxyacetophenone				
Total amount				
[μmol g^{-1} dry wt]	1.6	1.5	0.7	1.0
Distribution in %				
I	6	6	19	18
II	61	49	41	41
III	33	45	40	41
Hydroxybutenolide				
Total amount				
[μmol g^{-1} dry wt]	1.4	1.3	2.3	2.1
Distribution in %				
I	3	2	66	60
II	16	28	10	11
III	81	70	24	29
p-Hydroxybenzoic acid				
Total amount				
[μmol g^{-1} dry wt]	0.8	0.9	1.7	2.0
Distribution in %				
I	2	5	64	63
II	42	35	22	25
III	56	60	14	12
p-Coumaric acid				
Total amount				
[μmol g^{-1} dry wt]	0.3	0.3	0.8	0.7
Distribution in %				
I	6	7	85	84
II	25	23	6	5
III	69	70	9	11
trans-Cinnamic acid				
Total amount				
[μmol g^{-1} dry wt]	0.1	0.2	1.3	1.7
Distribution in %				
I	11	10	90	92
II	40	45	5	5
III	49	45	5	3

I: Buffer-soluble fraction.
II: Ethanol-extractable wall-bound fraction.
III: Dioxane-extractable wall-bound fraction.
*Plantlets collected from natural sites.
†Plantlets cultivated in bioreactors.

For isolation of the [*Z*]-3-(4′-hydroxyphenyl]-pent-2-en-1,5-dicarboxylic acid, for short, *cis*-sphagnum acid (**5**), we irradiated *trans*-sphagnum acid solutions with a 10-fold higher intensity and elucidated its structure by means of NMR spectroscopy. The NMR spectra of [*Z*]- and [*E*]-sphagnum acid showed identical numbers of signals and multiplicities, but significantly differing chemical shifts. All ^{1}H NMR signals were shifted high-field, especially those of protons H-4 ($\Delta\delta$ 0.72), which are opposite to the carboxylic C-1, away from its anisotropical area. On the other hand, the ^{13}C NMR signal of C-4 had a down-field shift, which is characteristic for *E*/*Z*-species of α, β-unsaturated carbonyl compounds [15]. Consequently, strong irradiation has to be avoided in experiments with sphagnum acid solutions and a calculation of the total amount of sphagnum acid must include both the

Table 2. Concentration of phenolics in the effluent media of *Sphagnum fallax* and *S. cuspidatum* cultivated in bioreactors

Phenolic compound	*S. fallax*	*S. cuspidatum*
trans-Sphagnum acid	20 nM	290 nM
cis-Sphagnum acid	2 nM	20 nM
trans-Sphagnum acid ethyl ester	3 nM	15 nM
Hydroxybutenolide	10 nM	130 nM
p-Hydroxybenzoic acid	5 nM	40 nM
p-Coumaric acid	10 nM	130 nM
trans-Cinnamic acid	0.1 nM	1.3 nM

trans and *cis*-isomer of this cinnamic acid derivative. Using HPLC, the *trans*- and *cis*-form are characterized by clearly different R_s, 19.5 and 10.5 min, respectively.

Compartmentalization

In the past, total *Sphagnum* plantlets, collected from natural sites, were frozen in liquid N_2, freeze-dried and used for studies on phenolics. As a result of such studies, sphagnum acid, hydroxybutenolide and *p*-hydroxybenzoic acid were described as components localized in the cell wall. Recently, some phenolics were also found to be excreted into the external medium of *Sphagnum* cultivated in bioreactors under axenic conditions; diverse phenolics were detected as endogenous free water-soluble compounds isolated from phosphate buffer homogenates prepared from living plant material [4, 16]. As a basis for studies on phenol metabolism in *Sphagnum*, we analysed the compartmentalization of sphagnum acid and the precursors *trans*-cinnamic acid and *p*-coumaric acid, as well as the peroxidative degradation products hydroxybutenolide, *p*-hydroxyacetophenone and *p*-hydroxybenzoic acid.

In Table 1, the measured amounts of these six phenolic compounds are documented for *S. magellanicum* and *S. fallax* collected from natural sites, as well as for *S. fallax* and *S. cuspidatum* cultivated in bioreactors. They are differentiated between the buffer-soluble phenolic fraction (I) and the amount of phenolics extractable from the cell walls with 96% ethanol (II) and dioxane–2 N hydrogen chloride (9:1) [III], respectively. Treatment with dioxane–hydrogen chloride leads to the solubilization of *ca* 80% of the cell wall material related to dry weight and the histochemical detection of cellulose is no longer masked. The data reveal that a hot ethanolic extraction is not sufficient to release the essential phenolics from the cell walls. After exhaustive ethanolic extraction, a dioxane–hydrogen chloride extraction, known as a reagent causing depolymerization by acidolysis, again yields high amounts of phenolics.

The most striking result is that sphagnum acid is predominantly buffer-soluble and is not localized mainly in the cell wall. Cultivation of *Sphagnum* in bioreactors leads even to an enhancement of its buffer-soluble part. *p*-Hydroxyacetophenone is predominantly bound to the cell wall and its buffer-soluble part in *S. fallax* cultivated in bioreactors is three fold higher. Hydroxybutenolide is also predominantly bound to the cell walls of *S. fallax* collected from natural sites; its buffer-soluble part is strongly enhanced in bioreactor plant material, from 2 to 66%. *p*-Hydroxybenzoic, *p*-coumaric and *trans*-cinnamic acids are predominantly localized in the cell wall, but in *S. fallax* derived from bioreactors a distinct enhancement of their buffer-soluble fraction is measureable. Thus, *S. fallax* cultivated in bioreactors is an important tool for the analysis of phenolic metabolism and compartmentalization studies.

However, studies on the secondary metabolism of *Sphagnum* are incomplete without considering the excretion of phenolics into the surrounding medium. In Table 2 the concentrations of phenolics in the effluent medium are given for *S. fallax* and *S. cuspidatum*, cultivated in bioreactors with a four-fold standard nutrient solution at a flow rate of 100 ml hr^{-1}. The phenolic concentrations in the effluent medium of *S. cuspidatum* were much higher than those of *S. fallax* related to the same fresh weight. In both cases, *trans*-sphagnum acid was dominant; *trans*-sphagnum acid ethyl ester and *cis*-sphagnum acid were also detectable, but in minor concentrations. It is noticeable that, although the buffer-soluble endogenous amount of *trans*-cinnamic acid was higher than that of *p*-coumaric acid (Table 1), the *p*-coumaric acid concentration is dominant in the effluent medium (Table 2). This rules out the possibility that excretion of phenolics into the effluent medium is due to leakage and is, therefore, only a diffusion phenomenon. Furthermore, studies on the biosynthesis of sphagnum acid with exogenously supplied labelled precursors demonstrated that these compounds are taken up actively from the medium and newly synthesized sphagnum acid is the first to be excreted [7].

EXPERIMENTAL

Plant material. Sphagnum magellanicum (Brid.) and *S. fallax* (Klinggr.) plantlets were collected in the Kaltenhofer Moor in Schleswig Holstein. *Sphagnum fallax* and *S. cuspidatum* (Hoffm.) were cultivated axenically in continuous feed bioreactors. The 4-fold concd standard nutrient soln was applied at a flow rate of 100 ml hr^{-1} [6, 16].

Soluble phenolic constituents. To determine the level of endogenous free H_2O-soluble phenolics, living plant material was homogenized in Pi-buffer (50 mM, pH 7) in a ratio of 1:5 (w/v) at 4°, using a cell homogenizer. The homogenate was filtered through gauze (80 μm). The residue, washed with Pi-buffer and twice with cold MeOH, was used for the determination of the cell wall-bound phenolics. The filtrate was centrifuged (30 min, 100 000 *g*), the supernatant filtered (2 μm membrane filter) and analysed by HPLC.

Cell wall-bound phenolic constituents. Washed residues were lyophilized and ball-milled. Cell walls were exhaustively extracted with boiling 96% EtOH at intervals of 24 hr. The remaining material was extracted with boiling dioxane–2 N HCl (9:1) at intervals of 24 hr. The EtOH and acidic dioxane extracts were centrifuged

(5000 *g*, 10 min), the supernatants neutralized, evapd to dryness and dissolved in 7% MeOH. The supernatants of centrifuged (10000 *g*, 15 min) solns were analysed by HPLC.

Phenolics in effluent media. The effluent media of *S. fallax* and *S. cuspidatum* cultivated in bioreactors were pumped at a flow rate of 100 ml hr^{-1} into cooled receiver bottles (0°), the pH adjusted to 7 and 500 ml of the medium concd in a rotary evaporator to 500 μl. The soln was centrifuged for 15 min at 10000 *g* and the supernatant analysed by HPLC.

Quantitative determination of phenolics. A HPLC chromatograph, 5 μm ODS II columns (4 × 150 mm), and a 20 μl sample loop was used for analysis. The solvent system was HCO_2H–H_2O (1:19) (A) and MeOH (B). The *A* was measured at 280 nm. The sepn program used was as follows: from 0 to 1 min B was held at 7% at a flow rate of 1 ml min^{-1}, from 1 to 29 min B was increased lineally to 45% at a flow rate of 0.6 ml min^{-1} and from 29 to 33 min from 48% to 95% at a flow rate of 1 ml min^{-1}.

Isolation of trans-*sphagnum acid ethyl ester and* p-*hydroxyacetophenone. Trans*-sphagnum acid ethyl ester and *p*-hydroxyacetophenone were isolated from EtOH extracts of *S. magellanicum* cell walls. Extracts were neutralized and evapd to dryness in a rotary evaporator. The residue was dissolved in 7% MeOH and centrifuged at 10000 *g*. Both phenolic compounds were sepd by HPLC with a semi-prep. column (ODS II, 5 μm). The frs containing the ester and *p*-hydroxyacetophenone, respectively, were evapd to dryness, dissolved in Me_2CO and their structure elucidated by means of NMR.

[E]-4-*Ethoxycarbonyl*-3-(4′-*hydroxyphenyl*)-*but*-2-*en*-1-*carboxylic acid* (trans-*sphagnum acid ethyl ester*). ^{1}H NMR (300 MHz, acetone-d_6): δ1.15 (3H, *t*, *J* = 7 Hz, (Me), 4.06 (2H, *q*, *J* = 7 Hz, OCH_2), 4.22 (2H, *s*, H-4), 6.26 (1H, *s*, H-2), 6.88 (2H, *m*, H-3′), 7.45 (2H, *m*, H-2′). ^{13}C NMR (75 MHz, acetone-d_6): δ14.4 (*q*, Me), 36.7 (*t*, C-4), 60.9 (*t*, OCH_2), 116.2 (*d*, C-3′), 117.6 (*d*, C-2), 128.9 (*d*, C-2′), 132.6 (*s*, C-3), 159.5 (*s*, C-4′), 167.6 (*s*, C-1), 170.5 (*s*, O–C=O).

4′-*Hydroxyphenyl-methyl ketone* (4-*hydroxyacetophenone*). ^{1}H NMR (300 MHz, acetone-d_6): δ2.56 (3H, *s*, Me), 6.99 (2H, *m*, H-3′), 7.96 (2H, *m*, H-2′), 9.1 (1H, *br*, OH). ^{13}C NMR (75 MHz, acetone-d_6): δ26.2 (*q*, Me), 115.8 (2C, *d*, C-3′), 130.3 (*s*, C-1′), 131.3 (2C, *d*, C-2′), 162.5 (*s*, C-4′), 196.2 (*s*, C=O).

Isolation of cis-*sphagnum acid. Cis*-sphagnum acid was prepd by irradiation (halogen lamp, 200 W m^{-2} s^{-1}, 1 hr) of a *trans*-sphagnum acid soln (10 μM, 7% MeOH). The *cis*-isomer was isolated by semi-prep. interval injection/displacement reversed-phase HPLC [17] and its structure elucidated by means of NMR.

[Z]-3-(4′-*Hydroxyphenyl*)-*pent*-2-*en*-1,5-*dicarboxylic acid.* ^{1}H NMR (300 MHz, acetone-d_6): δ3.0 (1H, *br*, OH), 3.51 (2H, *d*, *J* = 1 Hz, H-4), 6.02 (1H, *t*, *J* = 1 Hz, H-2), 6.78 (2H, *m*, H-3′), 7.21 (2H, *m*, H-2′). ^{13}C NMR (75 MHz, acetone-d_6): δ45.3 (*t*, C-4), 115.3 (2C, *d*, C-3′), 120.7 (*d*, C-2), 130.3 (2C, *d*, C-2′), 130.9 (*s*, C-1′), 150.9 (*s*, C-3), 158.2 (*s*, C-4′), 166.8 (*s*, C-1), 171.1 (*s*, C-5).

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