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Note

Simple screening method for the separation and identification of sphagnorubins, a new class of anthocyanidins

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With falling temperatures at the end of the summer, some peat mosses (*Sphagnaceae*) change their colour from green to deep red, thus giving whole moorlands a characteristic brown-red autumn look. In contrast to other plant pigments, the newly formed red dyes, named sphagnorubins[1], are located in the cell wall membranes[1,2]. Three of these membranochromes (sphagnorubin A, B and C) have been isolated and their structures elucidated[3,4]. They are anthocyanidins with a hitherto unknown 2-phenylphenanthro[2.1-*b*]pyran skeleton.

This paper presents easy methods for the identification of sphagnorubins, especially those chromatographic and spectroscopic properties which permit a rapid discrimination from other anthocyanidins. Using a simple procedure, several *Sphagnum* species were analysed for their content of the different sphagnorubins in order to establish whether the variations in the pigment composition can be used as a chemotaxonomic marker to discriminate peat moss species.

EXPERIMENTAL

Spectra

Absorption spectra were recorded with a Hitachi 220 double-beam spectrophotometer and fluorescence spectra with an Aminco-Bowman spectrophotofluorimeter. Cyanidine chloride (Roth, Karlsruhe, F.R.G.) served as reference anthocyanidin. Sphagnorubins were purified as described elsewhere[4].

Plant material

Red *Sphagnum* plants were collected in September or October in southern Sweden and F.R.G. (Schleswig-Holstein in the north or Bavaria in the south), air-dried immediately and analysed for pigment content within 4 weeks.

Thin-layer chromatographic method for identification and quantification of sphagnorubins

A 1-g amount (or less) of dried *Sphagnum* plants was pulverized with a mortar

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and extracted with methanol–0.5% concentrated hydrochloric acid until no red pigments eluted (usually 30–50 ml). Extraction was more effective at 55°C than at room temperature. The red extracts were concentrated to 1 ml with a rotary evaporator and 100-μl aliquots were spotted bandwise on cellulose thin-layer plates (Merck, Darmstadt, F.R.G.). The sheets were first developed with methanol–0.5% concentrated hydrochloric acid, air-dried and then developed a second time in the same direction with phenol–acetic acid–water–concentrated hydrochloric acid (15:80:20:1) until the front had travelled three quarters of the distance of the first run. The different sphagnorubins were identified by their R_F values (Table I). The corresponding red zones were scraped off and eluted with 2 ml of phenol–methanol–acetic acid–concentrated hydrochloric acid (10:80:20:1). The absorbance of the red solutions was measured at 540 nm *versus* blanks, and the pigment content was calculated from the molar absorptivities (Table I).

RESULTS AND DISCUSSION

The red moss pigments share many physical and chemical properties with anthocyanidins, the ubiquitous red and blue plant dyes. Several investigators[5–7] have previously noticed the similarity of the visible electronic spectra of the sphagnorubins and the common anthocyanidins (Fig. 1). Their absorption maxima are almost identical and they have high molar absorptivities (Table I). Moreover, the sphagnorubins undergo the same characteristic colour changes with pH; namely, red in acidic to blue in alkaline solution. However, whereas anthocyanidins such as cyanidin fade very rapidly in alkaline solutions, this process is slower for sphagnorubin B and C (Fig. 1), and sphagnorubin A is relatively stable under these conditions (spectrum nearly unchanged after 24 h). The pH-dependent colour changes of the sphagnoru-

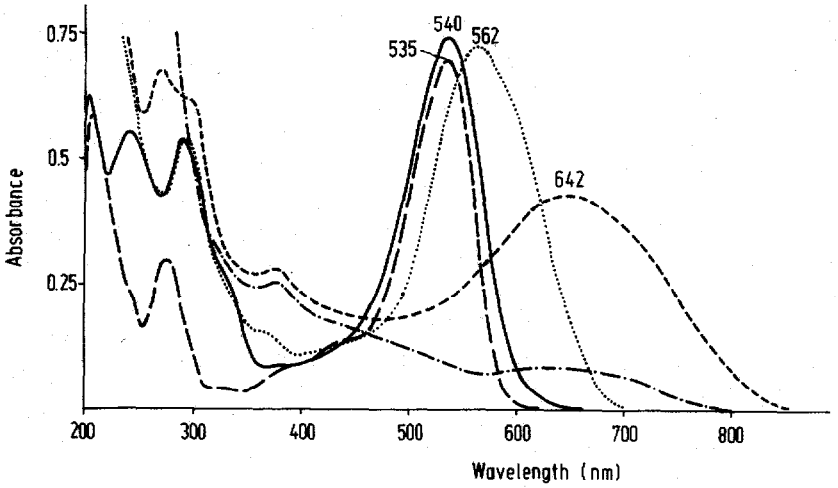


Fig. 1. Electronic spectrum of 1 μM sphagnorubin B in methanol–0.01% conc. HCl (———) in comparison to 2.6 μM cyanidin (- - - -). The spectral changes after addition of $AlCl_3$ (· · · · ·) and NaOH (- · - · -, immediately after mixing; - · - · - · -, after 1 h) are recorded. The spectra of sphagnorubin A and C are almost identical with those of pigment B (for visible maxima compare Table I), with the exception of the spectrum of sphagnorubin A in alkaline solution, which remains stable for at least 24 h.

TABLE I

SPECTRAL AND CHROMATOGRAPHIC PROPERTIES OF SPHAGNORUBINS IN COMPARISON WITH ANTHOCYANIDINS (CYANIDIN)

Property	*Sphagnorubin*			*Cyanidin*
	A	*B*	*C*	
Formula	1a	1b	1c	4
λ_{max} (nm) in methanol–0.01% conc. HCl	547	540	537	535
ε_{max} (l mol^{-1} cm^{-1})	75,000	75,000	75,000	28,000
λ_{max} (nm) after $AlCl_3$ addition	562	562	555	553
λ_{max} (nm) after NaOH addition	618	642	642	610
Fluorescence maximum, $\lambda_{excitation}$ (nm)	360	370	370	–
Fluorescence maximum, $\lambda_{emission}$ (nm)	423	412	410	–
R_F* (phenol–acetic acid–water–conc. HCl, 15:80:20:1)	0.10	0.25	0.45	1.0
R_F* (*n*-butanol–dimethylformamide–water–conc. HCl, 50:50:10:1)	0.30	0.36	0.41	1.0
R_F* (Forestal mixture**)	0.14	0.14	0.14	0.49
R_F* (Partridge mixture***)	0.08	0.08	0.08	0.68

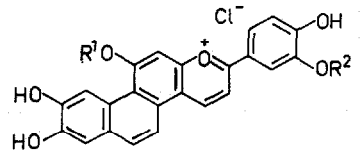
* R_F values on cellulose thin-layer plates.
** Acetic acid–water–conc. HCl (30:10:3).
*** *n*-Butanol–acetic acid–water (4:1:5, upper layer).

bins are assumed to correlate with structural changes from the red pyrylium salts (1a–c) in the acidic region through the blue anhydrobases (2a–c) to the colourless chalcones (3a–c) in the alkaline region, as discussed for the common anthocyanidins[8]. The anhydrobase 2a is a stable form of sphagnorubin A[3], as it crystallizes from pyridine in blue plates with this structure, and yields a corresponding derivative on acetylation[3]. In contrast, sphagnorubin B and C fade in pyridine and with acetic anhydride acetates of their stable chalcone structure (3b,c) are formed[4].

On addition of aluminium ions, parallel influences on sphagnorubins and common anthocyanidins are also observed: the moss pigments (Table I) exhibit a bathochromic shift (blue colour) like other anthocyanidins with catechol groups (*e.g.*, cyanidin[9,10]; Table I). Structural influences on the visible spectra are also similar, as small hypsochromic shifts in the maxima of sphagnorubin B and C compared with sphagnorubin A are also found for methylated anthocyanidins compared with the hydroxy-compounds[9]. In general, electronic spectra are not very useful for distinguishing between sphagnorubins themselves and to differentiate them from common anthocyanidins.

In contrast, fluorescence spectra are characteristic discriminators. Sphagnorubins are strong, blue fluorescent compounds (Fig. 2, Table I), when exposed to longwave ultraviolet light. This is probably an effect of the phenanthrene fluorophore (Fig. 2), which is absent from common anthocyanidins. Therefore, the latter are not fluorescent (except some glycosides and the coumaroyl derivatives[9]), and may thus easily be distinguished from sphagnorubins.

The most valuable method for distinguishing between sphagnorubins and common anthocyanidins is thin-layer chromatography on cellulose plates. Here, the solvent systems commonly used for the separation of anthocyanidins[9] such as Forestal mixture (acetic acid–concentrated hydrochloric acid–water, 30:3:10) or Partridge mix-



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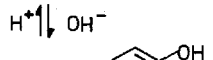

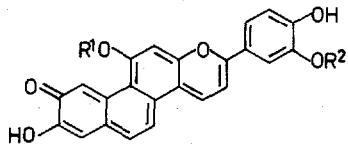

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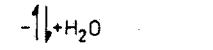
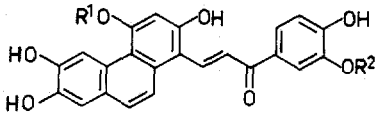
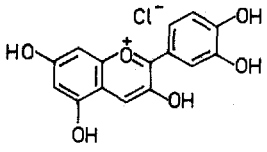




3

		Sphagnorubin
a	$R^1 = R^2 = H$	A
b	$R^1 = CH_3$ $R^2 = H$	B
c	$R^1 = R^2 = CH_3$	C



4 Cyanidin

ture (*n*-butanol–acetic acid–water, 4:1:5, upper layer) and other absorbents are unsuccessful for the chromatography of sphagnorubins, as they remain at or near the start. Only phenol- or dimethylformamide-containing solvents (Table I) effect a migration of sphagnorubins, whereas the mobilities of anthocyanidins are so high in these mobile phases that they are found in the solvent front.

Based on these distinctive chromatographic properties, we developed a simple two-step thin-layer method for the detection and identification of sphagnorubins in plant extracts. The pigments are solubilized from small amounts of *Sphagnum* plants (less than 1 g) with methanol–0.5% concentrated hydrochloric acid at room temperature or, more effectively, at 55°C. Aliquots of the concentrated extracts are spotted on cellulose thin-layer sheets. Development first with methanol–1% concentrated hydrochloric acid removes interfering compounds in a brown front zone, whereas

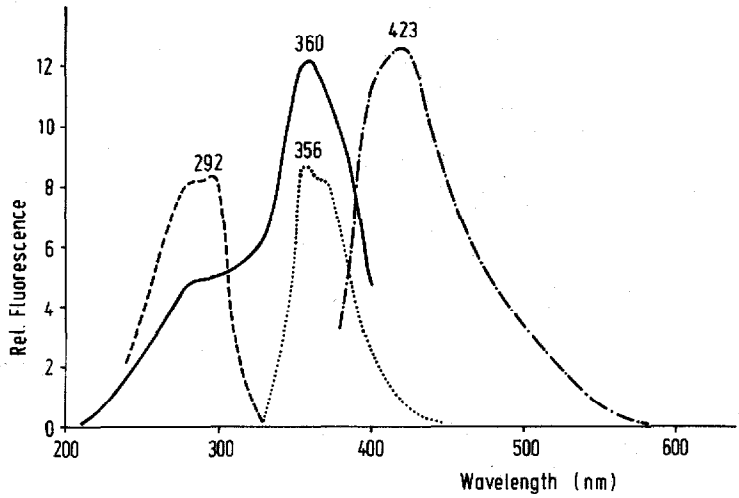


Fig. 2. Fluorescence spectra of spagnorubin A (———, excitation spectrum; - · - · - ·, emission spectrum) and phenanthrene (- - - -, excitation spectrum; · · · · ·, emission spectrum). The excitation and emission spectra are recorded at the corresponding emission and excitation maxima, respectively.

the red *Sphagnum* dyes remain at the start. After drying the plate, the different sphagnorubins are separated by chromatography with a second solvent (phenol–acetic acid–water–concentrated hydrochloric acid, 15:80:20:1) and identified by their R_F values (Table I) and blue fluorescence under longwave ultraviolet light. Quantification of sphagnorubins A, B and C is possible after scraping off the red zones, elution with phenolic solvents and absorbance measurement at 540 nm using the molar absorptivities given in Table I.

Table II summarizes the pigment composition of some *Sphagnum* species evaluated with this method. Although the species differ significantly in the proportions of the membranochromes, a smaller variation was also observed in plants of one species collected from different natural habitats (Table II). No changes in the

TABLE II

PIGMENT COMPOSITION OF SOME RED *SPHAGNUM* SPECIES

Sphagnum species	*Collected in*	*Concentration of total red pigments (μg/g dry moss)*	*Relative amounts (%) of sphagnorubins*		
			A	*B*	*C*
Sphagnum rubellum Wils.	Sweden, south	8	0	39	61
	F.R.G., north*	8	5	58	37
	F.R.G., south**	8	5	60	35
Sphagnum nemoreum Scop.	Sweden, south	8	5	5	90
Sphagnum plumulosum Röll	Sweden, south	4	17	34	49
Sphagnum magellanicum Brid.	F.R.G., north*	6	95	2–3	2–3
	F.R.G., south**	6	95	2–3	2–3

* Schleswig-Holstein.
** Bavaria.

pigment composition were found in the particular parts of the plants. Physiological aspects of the kinetics of the colouring with greenhouse material have been described elsewhere[11]. In spit of the observed variation of the pigment patterns, they may serve as an additional chemotaxonomic marker for the discrimination of the red peat mosses, of which many morphological varieties are known (*e.g.* from *S. rubellum* or *S. nemoreum*[12]). In addition, the simple chromatographic screening method may permit the detection of the remarkable sphagnorubins in other plants, as well as the rapid purification of small amounts of radiolabelled pigments in biogenesis studies.

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