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Moss Pigments

I. The Anthocyanins of *Bryum cryophilum* O. Mårt.

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Submitted in honour of the sixtieth birthday of our teacher, Professor *Arne Fredga*

Two anthocyanins have been isolated from *Bryum cryophilum* O. Mårt. They have been identified as luteolinidin-5-diglucoside and 5-monoglucoside.

Although the bryophytes form an important group in plant kingdom which is comparatively well explored from taxonomic and systematic points of view, little is known about their chemistry. As to their non-chlorophyllous pigments, which are of particular interest to the taxonomist as giving morphological characters, almost no detailed data regarding their chemical structure seem to have been available in the literature. However, the carotinoid distribution in several species, for example, has been qualitatively investigated recently [1].

Many of the earlier investigations of moss pigments refer to those of the anthocyanin type. Although none of these reddish or purplish pigments had been isolated and identified, their general chemical behaviour, such as their colour reactions, suggested that they are pigments belonging to the anthocyanin group.

Reddish pigments are widely distributed among the mosses and liverworts but those species in which this colour predominates in the general appearance of the plants are comparatively few. Among the "peat mosses" (the order Sphagnales) we have such definitely reddish-coloured species within the groups *Acutifolia* and *Palustria* and in Bryales we find them in genera such as *Bryum*, *Calliergon* and *Drepanocladus*. It should be pointed out, however, that the intensity of colouration may vary greatly within one species depending on nutrition and insolation conditions. These facts are well known to field bryologists and have been verified experimentally [2,3,4].

An extensive work on the occurrence of and the chemical behaviour of brownish and reddish pigments of bryophytes was carried out by Herzfelder [2]. She points out that the resulting colour of a moss or liverworts is a complex phenomenon as the pigmentation occurs in three different ways.

(1) The pigments occur in certain bodies in the cell, the chromatophores. The obligate chlorophyll and at least certain carotenes are fixed to such bodies.

(2) The pigments occur in the cell wall. A general characteristic is that they are often very difficult to extract completely. In addition to chlorophyll, the various cell wall pigments are the most important in the general colouration of moss plants. The cell wall pigments are generally more or less brownish (at least in old tissue) but may sometimes have a red to wine-red colour. This is particularly the case with the stem, the nerve and the border (when present) of the leaf, the cells towards the base of the leaf and the seta and capsula wall of the sporophyte. In some species, for example the genus *Sphagnum*, red cell wall pigments are very important and predominate in the colouration of the plants. According to Herzfelder's paper and our pre-investigations these pigments seem to be anthocyanidin derivatives although not of the same type as the anthocyanidin glycosides occurring in higher plants.

(3) Pigments may occur dissolved in the cell sap. As examples of species with such sap colouration, Herzfelder listed several *Bryum* species such as *B. duvalii* Voit* (a name now replaced by the correct *B. Weigelii* Spreng.), *B. pallens* Schleich., *B. schleicheri* Schwaegr. and *B. turbinatum* (Hedw.) Schwaegr. The reddish cell sap pigments of these species are said to be of anthocyanin type.

To find out whether or not *Bryum* species produce anthocyanins similar to those of the higher plants we have investigated the red pigmentation of *Bryum cryophilum* O. Mårt., a species earlier known under the invalid name *B. obtusifolium* Lindb. As earlier reported [5], two anthocyanins have been isolated from this moss and identified as luteolinidin-5-diglucoside and luteolinidin-5-monoglucoside.

B. cryophilum is a circumpolar species which occurs locally in abundance in arctic areas or mountain areas with an arctic climate. Generally it forms blood-red cushions or borders along small streamlets below snow areas. These investigations have been carried out with air-dried moss collected from the Torneträsk area of Torne Lappmark in northernmost Sweden as well as from Lake Peters in Brooks Range on the arctic slope of Alaska. No significant differences have been observed in the compositions of the anthocyanins in these materials. Provided the moss is carefully air-dried and preserved in a dark place, it may be stored for several years without notable destruction of the pigments. Since mosses are generally very resistant to dry periods — these may be considered as mere interruptions in the vegetation period — we did not consider it necessary to investigate the moss before it was dried. Unless used immediately, etiolation and other growing phenomena make such material less suitable for investigation than the dried material.

All red coloured material was precipitated by ether addition to the extract obtained by treating the air-dried and crushed moss at 0° with methanol

* The author's names have been added by the writers.

Table 1. Visible spectra of the anthocyanins, the corresponding aglycones and luteolinidin.

	λ_{max} in methanol-HCl (mμ)	λ_{max} in methanol-HCl−$AlCl_3$ (mμ)	$\frac{E_{440}}{E_{max}}$ (%)
Anthocyanin I	500	548	22
Aglycone I	498	549	38
Anthocyanin II	499	549	23
Aglycone II	499	549	37
Luteolinidin	499	548	38

containing 1 % of conc. HCl. However, the amount of anthocyanins in this precipitate was only about 10 %, the major part consisting of a complex mixture of yellow and dark-brown compounds.

The anthocyanins were separated from most of these by passing an aqueous acetic acid solution of the precipitate through a Sephadex column, the elution being performed with the same solvent. The two anthocyanins appeared as one orange-red band on the column and it was not possible to separate them from each other on this material. Three fractions were collected. The first one contained two different phenolic glycosides, the second the anthocyanins and the third a mixture of as yet unidentified compounds. The anthocyanin fraction contained at least five substances which were separated on a cellulose powder column with aqueous acetic acid as solvent. Anthocyanin I was obtained almost pure, whereas anthocyanin II had to be further purified by passing through another cellulose powder column before it was chromatographically pure.

It was also possible to isolate and purify the two anthocyanins by repeated chromatography on Whatman No. 3 MM filter paper using different solvents [6]. By this separation method a third red pigment occurring in *B. cryophilum* was also detected and isolated. Only traces of the latter have been obtained but preliminary investigations indicate that it might be an anthocyanidin derivative although not of a glycosidic type as the two isolated anthocyanins.

On acid hydrolysis of the two anthocyanins, two aglycones were obtained which proved to be identical. For example, they gave the same colour reactions,

Table 2. R_F values of the anthocyanins, the corresponding aglycones and luteolinidin.

	butanol-acetic acid-water 6:1:2	water-conc.HCl 97:3	butanol-2 N HCl 1:1 upper phase	acetic acid-conc.HCl-water 15:3:82	acetic acid-conc.HCl-water 30:3:10
Anthocyanin I	0.32	0.44	0.52	0.68	
Aglycone I		0.03			0.64
Anthocyanin II	0.41	0.13	0.31	0.37	
Aglycone II		0.03			0.63
Luteolinidin		0.02			0.63

their spectral and chromatographic properties were identical (Tables 1 and 2) and when co-chromatographed only one spot was obtained.

The R_F-value in water-acetic acid-conc. HCl (10:30:3, by vol.) indicated that the aglycone contains four hydroxyl groups [7] and the bathochromic shift of the maximum in the presence of aluminium chloride suggested that two or more of the hydroxyl groups are adjacent [8]. The aglycone has no hydroxyl group in the 3-position since it was not oxidized by dilute ferric chloride solution and, according to Robinson, only those anthocyanidins with an hydroxyl group in the 3-position are decolourised by this reagent [9].

The infrared spectrum of the aglycone showed all the characteristic features of an anthocyanidin spectrum [10,11]. The absence of a band at about 1460 cm^{-1} confirmed that the aglycone has no methoxyl group. Furthermore, the infrared spectrum was identical with that of luteolinidin (5,7,3′,4′-tetrahydroxyflavylium chloride) synthetized from the triacetate of phloroglucinaldehyde and 3,4-dihydroxyacetophenone diacetate [9,11].

Further evidence for identifying the aglycone as luteolinidin was obtained by comparing their spectral and chromatographic properties (Tables 1 and 2) as well as their colour reactions in acid and alkaline solutions [12]. Another proof of their being identical was that the co-chromatogram gave only one spot.

The position of attachment of the carbohydrates in the two anthocyanins was deduced from spectral data. According to Harborne [13], the percentage intensity at 440 mμ of a 5-substituted anthocyanidin is approximately half that of the corresponding anthocyanidin in which the 5-hydroxyl group is free. The value of the ratios E_{440}/E_{max} for the two anthocyanins as compared to that of the aglycone (Table 1) indicated that the isolated anthocyanins both have a substituted hydroxyl group in the 5-position.

Anthocyanin II was formed by controlled acid hydrolysis of anthocyanin I, the identification being based on chromatography and co-chromatography in different solvents [14]. This indicated that anthocyanin I is a diglycoside and anthocyanin II is the corresponding monoglycoside.

The sugars obtained on acid hydrolysis of the two anthocyanins were identified by paper chromatography in different solvents (Table 3) [15–17] as

Table 3. Identification of the sugars produced by hydrolysis of the anthocyanins.

	butanol [15]-acetic acid-water 4:1:5 upper phase 8 h	phenol [15] 10 h	pyridine [16]-butanol-water 7 h		ethyl acetate [17]-pyridine-water 2:1:2 5 h	
Glucose	0.175	0.42	0.385	0.42	0.54	0.64
Galactose	0.17	0.48	0.34	0.36	0.515	0.62
Fructose	0.25	0.555	0.45	0.47	0.57	0.65
Rhamnose	0.41	0.65	0.62	0.66	0.70	0.78
Sugar from anthocyanin I	0.17	0.42	0.39		0.545	
Sugar from anthocyanin II		0.42		0.41		0.66

glucose. Since the aglycone:glucose ratio in anthocyanin I was found to be 1:1.8, the two isolated anthocyanins must be luteolinidin-5-diglucoside and luteolinidin-5-monoglucoside.

Dr. O. Theander, Stockholm, has made a pre-investigation of the disaccharide obtained by hydrolysis of luteolinidin-5-diglucoside in dilute acetic acid. Although the amount of the disaccharide available was insufficient for a structure determination, the preliminary results indicated that the disaccharide part is 2-*O*-*α*-D-glucopyranosyl-D-glucose (kojibiose). As soon as more material is available this investigation will be continued.

Since the diglucoside is easily hydrolysed to the corresponding monoglucoside, it might be thought that the latter pigment is an artefact formed during the extraction procedure. However, since the relative amounts of the two glucosides are fairly independent of the time of extraction at 0° we suggest that the monoglucoside also occurs in the moss.

Luteolinidin has long been known as a synthetic compound but has not until recently been found to occur naturally as luteolinidin-5-monoglucoside in the petals of *Gesneria cardinalis* and in the sepals and leaves of *Kohleria eriantha* (fam. Gesneriaceae)[18]. Luteolinidin-5-diglucoside has not earlier been found in nature.

It may be pointed out that *B. cryophilum* is considered to be closely related to *B. duvalii* investigated by Herzfelder and found to contain cell sap anthocyanins. Our investigation indicates that *B. cryophilum* contains not only cell sap anthocyanins which are identical with the luteolinidin derivatives isolated but probably also cell wall pigments. This suggestion is substantiated by the fact that it is impossible to extract all the red pigments by merely treating the moss in the cold with methanolic hydrochloric acid. Several hours refluxing with the solvent is necessary to free the remainder from red pigments. The same difficulties connected with the complete extraction of cell wall pigments have been earlier pointed out by Herzfelder and recently by Goodman and Paton when investigating some *Sphagnum* species. This agrees with our extraction experiments on some *Sphagnum* species showing that the cell wall pigments are only partially extracted in the cold by the above mentioned solvent.

Luteolinidin, luteolinidin-5-monoglucoside and the third pigment have been found in the methanolic extract obtained by boiling moss previously extracted in the cold. This fact suggests that some luteolinidin derivative, possibly one or both of the isolated anthocyanins, may also occur fixed in some way to the cell wall. As to the third pigment we have observed a certain resemblance between it and some red cell wall pigments isolated from two *Sphagnum* species suggesting that the former might occur in the cell wall only.

Although only a single moss with cell sap pigments has been investigated, there are reasons to believe that either luteolinidin derivatives or similar anthocyanins may also occur in closely related species of the same genus with cell sap pigments. Regarding the mosses containing only cell wall pigments, our pre-investigations of the red pigments of *Sphagnum magellanicum* Brid. and *S. nemoreum* Scop. (belonging to the groups *Palustria* and *Acutifolia*, respectively) indicate that they are different to the anthocyanins from *B. cryophilum*. At least one of the red pigments in each of these *Sphagnum* species

seems to be an anthocyanidin derivative although not a glycoside since preliminary assays indicate that these two isolated pigments do not contain sugars.

We have also investigated a pleurocarpous moss, *Drepanocladus pseudosarmentosus* (Card. & Thér.) H. Perss., which has reddish cell wall pigmentation. One of the pigments of this moss shows a chemical behaviour somewhat resembling that of the red pigments of anthocyanidin type isolated from the two *Sphagnum* species mentioned above. The other pigment seems to be related to the anthocyanins of *B. cryophilum*.

EXPERIMENTAL

Whatman No. 1 filter paper was used for the paper chromatography unless otherwise stated. The descending chromatographic method was always used. The absorption spectra were measured in methanol containing 0.01 % conc. HCl with a Beckman DU spectrophotometer and the infrared absorption measurements were made with an Unicam 100 spectrophotometer with a sodium chloride prism.

Separation and purification of the anthocyanins. Air-dried moss (22 g) was chopped in a Turmix blendor in methanol-conc. HCl (35:1 v/v) (400 ml), kept at 0° overnight and filtered. Fresh solvent (150 ml) was added to the moss and after a week's storage at 0° it was again filtered. The precipitate formed on addition of ether (15 times the volume) to the combined methanolic extracts was treated with the smallest possible amount of aqueous acetic acid (10 %) and insoluble material removed by centrifugation. The acetic acid solution was passed through a Sephadex (Pharmacia, Uppsala), column, the elution being performed with the same solvent. Three fractions were collected. The second contained the anthocyanins and was evaporated to dryness *in vacuo*. The residue was taken up in aqueous acetic acid (5 %) and chromatographed on a cellulose powder (Grycksbo) column, the same solvent being used for the elution. The eluate was collected in 10 ml fractions and its absorption was recorded at 253 mμ by means of an ultraviolet registrating photometer. Five distinct bands were obtained of which No. 2 and No. 4 contained anthocyanins I and II, respectively. The fractions corresponding to band No. 2 were combined and evaporated to dryness *in vacuo* and the residue (120 mg), consisting of anthocyanin I, was recrystallized from methanol-conc. HCl yielding dark reddish-brown crystals. The fractions containing anthocyanin II were concentrated *in vacuo* to a small volume and again chromatographed on a cellulose powder column as above. After evaporation to dryness *in vacuo* a dark red residue (4 mg) was obtained which was chromatographically pure.

For separation and purification by means of paper chromatography the combined methanolic extracts were concentrated to a small volume *in vacuo* at 30–40°, extracted with petroleum ether and ethyl acetate and finally diluted with methanol-conc. HCl (97:3 v/v). After centrifugation the methanolic solution was applied as a thin band near one edge of a Whatman No. 3 MM filter paper. When developed with the upper phase of butanol-2 N HCl (1/1 v/v) three red bands were obtained, corresponding to anthocyanins I and II and the third red pigment. These were cut out without previous drying and the pigments were eluted with water-methanol-acetic acid (25:70:5, by vol.). Each of the resulting solutions were then concentrated to a small volume *in vacuo*, applied as a streak on No. 3 MM Whatman filter paper and further purified by running in water-acetic acid (85:15 v/v), the elution being made as before. The last traces of impurities were removed by re-running the anthocyanins in the same solvent. Since only a small amount of the third pigment was obtained this could not be satisfactorily purified.

The isolated anthocyanins were tested for homogeneity by paper chromatography in different solvents [19]. The R_F values are given in Table 2 and the spectral data in Table 1.

Identification of the anthocyanins. (*a*) Qualitative hydrolysis. The glucosides were heated with 4 N HCl at 100° for one hour and the solution was then cooled. The aglycones were extracted with pentanol. The combined extracts were washed with water and after removal of the solvent the aglycones were dissolved in methanol-conc. HCl (97:3 v/v). The R_F values, colour reactions and the absorption spectra of the two aglycones were

identical to those of a synthetic sample of luteolinidin (Tables 1 and 2). The infrared spectrum (KBr phase) of the aglycone obtained from anthocyanin I was also identical with that of luteolinidin (KBr phase).

The aqueous phases and the washings from the hydrolysis experiments were combined in order to analyse the sugars and the acid removed by washing with 10 ml portions of a 10 % (w/w) solution of dioctylmethylamine in chloroform until the aqueous solutions were neutral [20]. The aqueous solutions were then concentrated *in vacuo* and chromatographed together with authentic markers in four different solvent systems [15-17]. The sugars were detected by spraying the chromatograms with aniline hydrogen phthalate in butanol and heating at 110° for 5 min.[21] The results are shown in Table 3. The two hydrolysis solutions always yielded a single spot corrresponding to glucose.

(*b*) Quantitative hydrolysis [22]. Anthocyanin I (*ca.* 2.5 mg) in 4 N HCl (1.5 ml) was heated for 30 min in the dark (to prevent decolourization) at 100° under reflux, cooled and diluted with water to 10 ml. The absorbance of this solution at 480 mμ was immediately measured and by means of a standard solution, measured in the same solvent, the concentration of the aglycone was determined.

For determination of the sugar concentration 1 ml of the solution used for the above measurements was added to an anthrone solution (10 ml 0.2 % in 95 % sulphuric acid) and immediately heated for 7 min on a boiling water bath, 10 min after the heating had ceased, the absorption of the solution was measured at 620 mμ and compared with standard glucose solutions [23]. The following results were obtained:

concentration of aglycone:	0.11 mg/ml
» » glucose:	0.12 mg/ml
molar ratio glucose/aglycone:	1.8 : 1

(*c*) Partial hydrolysis of anthocyanin I. Anthocyanin I (2 mg) in 2 N HCl (3 ml) was heated at 100° for 10 min. The mixture was cooled, applied as a streak to No. 3 MM filter paper and developed with the upper phase of butanol-2 N HCl (1:1 v/v). Three red bands were obtained, cut out and eluted with water-methanol-acetic acid (25:70:5 by vol.). The concentrated eluates were re-run in water-acetic acid conc. HCl (82:15:3 by vol.). After elution the pigments were identified as unchanged anthocyanin I, anthocyanin II and luteolinidin by means of chromatography and co-chromatography.

Determination of the disaccharide. Anthocyanin I (20 mg) was refluxed for 5 h with aqueous acetic acid (10 %; 5 ml). After cooling, the solution was extracted with pentanol and the extract washed with water. The acetic acid solution and the washings were evaporated to dryness *in vacuo*. The residue, containing only a very small amount of the disaccharide, has been investigated by Dr. O. Theander, Svenska Träforskningsinstitutet, Träkemiska avdelningen, Stockholm. However, the amount of the disaccharide was not sufficient for a complete determination of the structure.

Investigation of the extract obtained by refluxing the moss. Dried moss extracted twice at 0° with methanol-conc. HCl (35:1 v/v) was refluxed for 4 h with the same solvent and then filtered. After concentration *in vacuo* to a small volume and treatment with petroleum ether and ethyl acetate, the methanolic solution was applied to Whatman No. 3 MM filter paper. When developed with the upper phase of butanol-2 N HCl (1:1 v/v), three red bands were obtained. These were cut out, eluted with water-methanol-acetic acid (25:70:5 by vol.) and concentrated *in vacuo* at 30—40° to a small volume. By chromatographic data the three pigments were identified as luteolinidin, luteolinidin-5-monoglucoside and the third red pigment.

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