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LUNULARIC ACID AND RELATED COMPOUNDS IN LIVERWORTS, ALGAE AND *HYDRANGEA*

JOHN GORHAM*

Dept. of Horticulture, Wye College, University of London, Nr. Ashford, Kent, TN25 5AH, England

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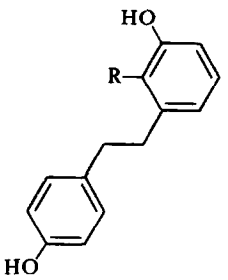
Key Word Index—*Hydrangea macrophylla*; Saxifragaceae; Algae; Hepaticae; liverworts; stilbenes; bibenzyls; lunularic acid; lunularin; chemotaxonomy.

Abstract—Lunularic acid and lunularin were detected in 76 species of hepatics, but not in any of the Anthrocerotales or Algae examined. Lunularic acid, lunularin, 3,4′-dihydroxystilbene and a glycoside of lunularic acid were also identified in extracts of *Hydrangea macrophylla* roots, together with hydrangenol, hydrangeic acid and their glucosides.

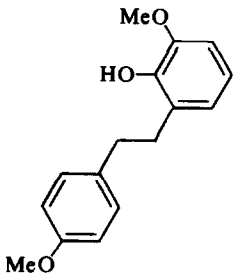
INTRODUCTION

Lunularic acid (3,4′-dihydroxybibenzyl-2-carboxylic acid) **1** was first reported as a growth inhibitor and dormancy factor in the Israel strain of the liverwort



1 R = COOH
2 R = H

Lunularia cruciata [1]. It has since been reported in a number of hepatics [2–4] and in Algae [3], but not mosses, ferns or higher plants, with the exception of a bound form in *Hydrangea macrophylla* (Saxifragaceae) [5] in which the related compounds hydrangenol (**6**), hydrangeic acid (**4**) and their glucosides have been detected. The decarboxylation product of lunularic acid, lunularin (3,4′-dihydroxybibenzyl) **2**, has also been identified in extracts of *Lunularia cruciata* [6] and *Marchantia polymorpha* [7] and in the heartwood of *Morus laevigata* (Moraceae) [8, 9]. These two compounds, together with pellepiphyllin (3,4′-dimethoxy-2-hydroxybibenzyl) **3**, occur in *Pellia neesiana* [6, 10], 2,3,4′-trihydroxybibenzyl in *Pellia endiviifolia* [11], 3-methoxybibenzyl in *Radula complanata* [12] the Brittonins (3,4,5,3′,4′,5′-hexamethoxybibenzyl and 3,4,5,3′-tetramethoxy-4′,5′-methylenedioxybibenzyl) in *Frullania brittoniae* subsp. *truncatifolia* [22] and these are the only stilbenoid compounds that have been detected in lower plants. This paper describes a more extensive survey of the distribution of



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lunularic acid and lunularin in liverworts and algae and examines the stilbenoid composition of *H. macrophylla*.

RESULTS AND DISCUSSION

Lunularic acid has previously been extracted with ethanol either in a soxhlet apparatus [2] or by soaking the material at low temperature [1]. More efficient extraction could be obtained by refluxing the tissue with 2N methanolic HCl for 1 hr. Under these conditions no thermal decarboxylation of pure lunularic acid solutions could be detected by GLC. Furthermore, no acid-labile, bound forms of lunularic acid could be extracted from *Conocephalum conicum* with acetone, methanol or water. The methanolic HCl extraction procedure was therefore used throughout the present investigation.

For the identification of lunularic acid and lunularin from new plant sources preliminary TLC purification was used before subjecting the extracts to GLC analysis (Table 3). However, when quantitative determinations of lunularic acid from thalloid liverworts were required, the silylated crude combined acids fraction was injected directly onto the GLC columns since experiments with ^{14}C-labelled lunularic acid had revealed that a large proportion (up to 50%) of the lunularic acid was lost when extracts were chromatographed on thin layers of silica gel. Silylation with BSA was more convenient than methylation with diazomethane [2] for the production of volatile derivatives of lunularic acid and lunularin, and

*Present address—Dept. of Biological Sciences, Portsmouth Polytechnic, King Henry 1st Street, Portsmouth, PO1 2DY.

produced a greater FID response. Using these techniques lunularic acid could be reliably detected in 10 mg fr. wt samples of thallose liverworts.

The species of hepatics examined for lunularic acid and lunularin are listed in Table 1. Both compounds were detected in all the species examined (except the Anthocerotales), at levels ranging from less than 1 μg/g fr. wt lunularic acid and lunularin in some members of the Jungermanniales to 200–650 μg/g fr. wt lunularic acid and 10–20 μg/g fr. wt lunularin in *Conocephalum conicum*, depending on the conditions in which the plants had been grown. Thalli grown in high light intensities (5600lx) contained higher levels of lunularic acid than those cultured in low intensity light (140 or 560 lx), and more lunularic acid was found in plants grown in long days than in those subjected to short day conditions. Lunularic acid was detected in all parts of the liverworts—in antheridiophores and archegoniophores of *Preissia quadrata*, sporophytes of *Pellia epiphylla*, rhizoids of *Marchantia polymorpha* and gemmae of *Lunularia cruciata*. The highest concentrations were found at the apex of the thallus.

Table 2 lists the species of Anthocerotales, algae, lichens, mosses and ferns that were examined. Some of these extracts, after TLC purification, produced GLC peaks of the same R_t as lunularic acid–TMSi on either

Table 1. Liverworts in which both lunularic acid and lunularin have been detected

Order*	Family	Species†
MARCHANTIALES	Aytoniaceae	*Asterella venosa* (Lehm. & Lindenb.) Evans
		Mannia fragrans (Balbis) Grye et L.
		Mannia capensis (Steph.) Arn.
		Plagiochasma crenulatum Gottsche
		Plagiochasma rupestre (Forst.) Steph.
		Plagiochasma australe Nees
		Reboulia hemisphaerica (L.) Raddi
	Conocephalaceae	*Conocephalum conicum* (L.) Underwood
		Conocephalum supredecompositum (Lindenb.) Steph.
	Corsiniaceae	*Corsinia coriandrina* (Spreng.) Lindb.
	Exormothecaceae	*Exormotheca bullata* (Link) K.M.
	Lunulariaceae	*Lunularia cruciata* (L.) Dum.
	Marchantiaceae	*Marchantia alpestris* (Lees) Burgeff
		Marchantia berteroana Lehm. & Lindb.
		Marchantia cataractarum var. *luzonica* Burgeff
		Marchantia paleacea Bertoloni
		Marchantia planiloba Steph.
		Marchantia palmatoides Burgeff
		Marchantia sp. Kew No. 247-70 02326 Jermy 6752
		Dumortiera hirsuta (Schwartz) Nees
		Dumortiera velutinum Schiff.
		Preissia quadrata (Scop.) Nees
		Wiesneriella denudata (Mitt.)
	Monocleaceae	*Monoclea fosteri* Hook.
	Oxymitraceae	*Oxymitra paleacea* Bisch.
	Ricciaceae	*Riccia fluitans* L.
		Riccia gangetica Ahmad.
		Riccia angolensis Stephani
		Riccia ciliifera Link
	Sphaerocarpaceae	*Sphaerocarpos michelii* Bellardi
	Targioniaceae	*Targionia hypophylla* L.
METZGERIALES	Aneuraceae	*Riccardia latifrons* (Lindb.) Lindb.
		Riccardia pinguis (L.) Gray
		Riccardia multifida (L.) Gray
	Blasiaceae	*Blasia pusilla* L.
	Codoniaceae	*Fossombronia angulosa* (Dicks.) Raddi
	Metzgeriaceae	*Metzgeria furcata* (L.) Dum.
	Pelliaceae	*Pellia endiviifolia* Dicks.
		Pellia epiphylla (L.) Corda
		Pellia neesiana (Gottsche) Limpr.‡
		Moerckia blyttii (Mörch) Brockm.
CALOBRYALES	Haplomitriaceae	*Haplomitrium rotundifolium* (Mitt.) Schiff.
		Haplomitrium gibbsiae Steph.
JUNGERMANNIALES	Adelanthaceae	*Odontoschisma sphagni* (Dicks.) Dum.
	Antheliaceae	*Anthelia julacea* (L.) Dum.
	Cephaloziaceae	*Cephalozia bicuspidata* (L.) Dum.
		Nowellia curvifolia (Dicks.) Mitt.
	Calypogeiaceae	*Calypogeia fissa* (L.) Raddi
	Gymnomitriaceae	*Marsupella emarginata* (Ehrh.) Dum.
	Jubulaceae	*Frullania dilatata* (L.) Dum.
		Frullania tamarisci (L.) Dum.

Table 1 continued

Order	Family	Species
	Jungermanniaceae	*Leiocolea muelleri* (Nees) Jorg.
		Lophozia incisa (Schrad.) Dum.
		Mylia taylori (Hook.) Gray
		Mylia anomala (Hook.) Gray
		Nardia compressa (Hook.) Gray
		Nardia scalaris (Schrad.) Gray
		Solenostoma crenulatum (Sm.) Mitt.
		Solenostoma triste (Nees) K. Müll.
	Lepidoziaceae	*Bazzania trilobata* (L.) Gray
		Lepidozia reptans (L.) Dum.
	Lophocoleaceae	*Lophocolea cuspidata* (Nees) Limpr.
		Lophocolea heterophylla (Schrad.) Dum.
	Plagiochilaceae	*Plagiochila asplenioides* var. *asplenioides* (L.) Dum.
		Plagiochila spinulosa (Dicks.) Dum.
	Pleuroziaceae	*Pleurozia purpurea* Lindb.
	Porellaceae	*Porella platyphylla* (L.) Lindb.
	Radulaceae	*Radula complanata* (L.) Dum.
	Scapaniaceae	*Diplophyllum albicans* (L.) Dum.
		Scapania aspera Bernet
		Scapania gracilis (Lindb.) Kaal.
		Scapania irrigua (Nees) Dum.
		Scapania nemorea (L.) Grolle
	Trichocoleaceae	*Trichocolea tomentella* (Ehrh.) Dum.

*Classification according to Grolle. †Specific names of British species given in Census Catalogue of British Hepatics (Ed. Paton, J. A.). British Bryological Society, 1965. ‡Pellepiphyllin was also detected.

SE-30 or OV-17 columns, and even when lunularic acid–TMSi was added to the extract only one peak was produced. However, these peaks were shown not to be due to lunularic by use of one or both of the other columns.

A chemotaxonomic distinction between the Anthocerotales and the other hepatics has already been reported in respect of their metabolism of the D-isomers of amino acids [13]. Whereas most hepatics deaminate D-methionine, *Anthoceros* produced the *N*-malonyl conjugate—a feature of higher plant metabolism. This group of liverworts is now also seen to differ from the rest of the Hepaticae by the absence of lunularic acid. Thus the biochemical evidence supports the status of the Anthocerotales as a completely separate class, the Anthocerotae [14, 15], rather than an order within the class Hepaticae [14].

The inability to detect lunularic acid in algae once more raises the question of the occurrence of abscisic

Table 2. Species in which lunularic acid was not detected

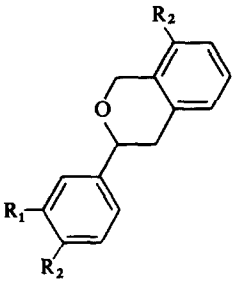
Anthocerotae	Anthocerotales	*Anthoceros laevis* L.
		Anthoceros punctatus L.
		Phaeoceros sp.*.
Mosses	Eubryales	*Mnium hornum* Hedw.
		Mnium undulatum Hedw.
	Fissidentales	*Fissidens taxifolius* Hedw.
		Fissidens adiantoides Hedw.
	Pottiales	*Tortula ruralis* (Hedw.) Crome
	Dicranales	*Dicranella heteromalla* (Hedw.) Schimp.
	Sphagnales	*Sphagnum palustre* L.
Algae†	Chlorophyceae	*Chlorella* sp.
		Ulva lactuca L.
	Phaeophyceae	*Fucus vesiculosus* L.
		Sargassum muticum (Yendo) Fensholt.
	Cyanophyceae	*Anabaena inaequalis* (Kötz) B. and F.
	Xanthophyceae	*Monodus subterraneus* Petersen
Ferns	Filicales	*Phyllitis scolopendrium* (L.) Newm.
		Asplenium adiantum-nigrum L.
Lichens		*Xanthoria parietina* Th. Fr.
		Peltigera canina Willd.

*Air-dried samples supplied by Dr. Bhatadwaj. †5g–1 kg fr. wt.

acid in this group of plants. It was originally supposed [2] that there was a mutually exclusive distribution pattern of lunularic and abscisic acids in plants, with lunularic acid occurring in algae and liverworts and abscisic acid in mosses, ferns and higher plants. However, despite earlier reports of the absence of abscisic acid in algae [3], the work of Hussain and Boney [19] suggested the presence of abscisic acid in *Laminaria* and *Ascophyllum*. There is also some evidence for the presence of other acidic growth inhibitors in algae [17, 18].

Pryce [5] has reported the presence of an acid-labile bound form of lunularic acid in the roots of *Hydrangea macrophylla*. In order to investigate the possibility that this is a glucoside, 2 g of washed *Hydrangea* roots were incubated in the dark for 16 hr with lunularic acid labelled universally in its phenylpropanoid moeity with ^{14}C (200000 dpm). Whilst most of the extractable radioactivity was found in the strong acid fraction (53 100 dpm), a large proportion was detected in the amyl alcohol soluble fraction (13 900 dpm). When this fraction was chromatographed on polyamide–silica gel (1:1) developed in isobutanol–methanol–water (80:6:15) only one radioactive band was obtained, and this produced lunularic acid (detected by GLC) on acid hydrolysis or after incubation with β-glucosidase. Methylation of part of the amyl alcohol soluble fraction followed by acid hydrolysis, TLC and silylation resulted in a peak corresponding to 3-hydroxy-4'-methoxy bibenzyl-2-carboxylic acid–TMSi on both OV-17 and SE-30 columns, suggesting that the bound form of lunularic acid in *Hydrangea* might be the 3-β-D-glucopyranoside, although confirmation of this must await the isolation of pure, crystalline material.

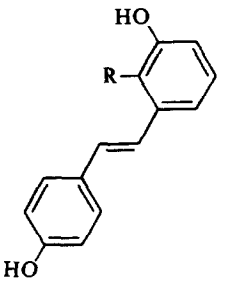
Extraction of the roots of *Hydrangea* with acetone and subsequent fractionation yielded an amyl alcohol soluble fraction containing the bound form of lunularic acid together with the glucosides of hydrangenol and hydrangeic acid (identified by TLC, UV spectra and hydrolysis to the aglycones) and a combined acids fraction which contained hydrangenol, hydrangeic acid, lunularic acid and lunularin as determined by TLC and GLC. The decarboxylation product of hydrangeic acid, 3,4'-dihydroxystilbene (**5**), was also detected for the first time



6 R_1 = H, R = OH
7 R_1 = OH, R = OCH_3

in these extracts by co-TLC and GLC with a synthetic sample. The quantities of lunularic acid, lunularin and 3,4'-dihydroxystilbene present in these extracts (< 1 μg/g fr. wt) would not have been detected by TLC or PC alone.

No lunularic acid or hydrangenol was detected in *H. quercifolia* [5], nor could hydrangenol be detected in several other species of *Hydrangea* [20]. Hydrangenol and phyllodulcin (**7**) from *H. macrophylla* var *thunbergii* are the only known naturally occurring 3-phenyldihydroisocoumarins.



4 R = COOH
5 R = H

EXPERIMENTAL

Lunularic acid—[^{14}C] was prepared as in ref. [5], except that thallus tips of *Conocephalum* were used.

Radioactivity measurements. A liquid scintillation counter was used to determine the radioactivity of the soluble samples and TLC-radioautographs of 50 × 200 mm TLC plates were obtained with a scanner.

Chromatography. TLC was carried out on 250 or 500 μm thick layers of Si gel GF_{254} and spots were eluted with MeOH. A dual column GLC fitted with FIDs was used with 1.5 m × 4 mm id silanized glass columns packed with 1% OV-17, 1.5% XE-60 or 3% SE-30. Chromatographic data are given in Table 3.

Extraction. For extraction of lunularic acid plant material was refluxed in 2N HCl in MeOH for between 1 and 4 h, depending on the nature and quantity of the material. After filtration through Celite 545 and evaporation of the MeOH *in vacuo*, a strong acid fraction was obtained as described below. MeOH extracts of other plant materials were evaporated *in vacuo* and the aq. residue partitioned 3× with Et_2O after acidification of the residue to pH 2 with conc HCl. The aq phase was further partitioned with amyl alcohol to extract the stilbene glycosides. Extraction of the Et_2O phase with 5% Na_2CO_3 removed a strong acids fraction and this was followed by extraction with N NaOH to give a weak acids fraction and a residual neutral fraction. If NaOH alone was used the resulting aq phase was referred to as the combined acids fraction. These fractions were extracted into Et_2O after acidification with HCl to pH 2.

Synthesis of stilbenes. 3,4'-Dihydroxystilbene was prepared by Wittig condensation of diEt(4-methoxybenzyl)phosphonate with 3-methoxybenzaldehyde followed by fusion of the dimethoxystilbene with pyridinium chloride. Reduction of 3,4'-dihydroxystilbene with H_2 in the presence of 5% Pd/C gave lunularin (mp 106–108°; Lit. 105–107° [6]; λ_{max}^{MeOH} (nm) 275, 279; λ_{max}^{MeONa} (nm) 288). Decarboxylation with Cu/quinoline of 3,4'-diacetoxystilbene-α'-carboxylic acid (produced by Perkin condensation of Na(4-hydroxyphenyl)acetate and 3-hydroxybenzaldehyde in Ac_2O), followed by hydrolysis with NaOH–EtOH under N_2, also produced 3,4'-dihydroxystilbene, but in smaller yield.

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Table 3. TLC and GLC data of lunularic acid and related compounds.

Compound	TLC R_f EtOAc–$CHCl_3$–HOAc (15:5:1)	TLC R_f C_6H_6–MeOH–HOAc (20:4:1)	TLC R_f HOAc–$CHCl_3$ (9:1)
Lunularic acid	0.50	0.33	0.75
Lunularin	0.64	0.38	0.75
Hydrangeic acid	0.45	0.22	0.68
Hydrangenol	0.73	0.40	0.80
3,4′-Dihydroxystilbene	0.70		

Compound	3% SE-30*			1% OV-17†			1.5% XE-60†		
	R_t	temp	RI	R_t	temp	RI	R_t	temp	RI
Lunularic acid–TMSi	12.1	243°	2555	8.5	206°	2725	9.6	190°	2815
Lunularin–TMSi	4.6	243°	2215	3.7	196°	2330	2.2	190°	2372

*N_2 flow rate 40 ml/min. †N_2 flow rate 60 ml/min.

and Mrs. A. G. Side for liverwort material and Mr. W. F. Farnham (Portsmouth Polytechnic) for air-dried material of *Sargassum muticum*. Lunularic acid and 3-hydroxy-4′-methoxybibenzyl-2-carboxylic acid were kindly supplied by Hoffman-La Roche. Mr. B. D. Thomas carried out part of the extraction work with algae. My thanks are due to Prof. W. W. Schwabe for advice and encouragement and to Dr. A. R. Perry for herbarium facilities. During the course of this work, which was carried out in partial fulfilment of the requirements for the degree of Ph.D. of the University of London, the author held an SRC studentship and a Riley-Luxton Scholarship.

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