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CHEMOTAXONOMY IN DRYOPTERIS AND RELATED FERN GENERA

Review and evaluation of analytical methods

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Abstract

The most important phloroglucinol derivatives ("filicins") isolated from rhizomes of *Dryopteris* species are discussed. The chemical analysis of *Dryopteris* rhizomes can (in conjunction with classical and cytological methods) provide valuable criteria for the solution of taxonomical problems, particulary as follows: 1. Identification of otherwise uncertain herbarium specimens. 2. Classification of critical taxa in a difficult complex. 3. Understanding of natural relations, including parents in hybrides and ancestors in allopolyploid species. Analytical methods are critically evaluated.

1. **Introduction.** The chemical composition of a plant or part of it (seed, flower, leaf, rhizome, root etc.) can be of valuable help in solving taxonomic problems and is today widely accepted as a (potentially) useful indicator. It should, however, be evaluated critically and as only one character in combination with classical (morphological, anatomical and cytological) data. In the chemical approach the analysis of primary products (nucleic acids and proteins) should give the most reliable basis but requires complicated methods and expert training. In practice the analysis of secondary products (e.g. alkaloids, flavanoids, anthocyanins versus betacyanins, different types of glycosides, sulfur compounds, asetylenes, carotinoids, etc.) is often more convenient. For reviews see R. Hegnauer (1962-1973); T. Swain (1963); J.B. Harborne (1973); A. Hiraoka (1978).

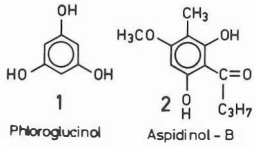
Use of chemical characters for taxonomical purposes is in principle not a recent invention if we consider that registration of colour, fragrance, and bitter or other taste, are simple but often useful first approaches to "chemical analysis", based on sense perception and practised since older times. For more precise information even a small amounts of material, very efficient modern analytical methods are available: paper chromatography (PC), thin layer chromatography (TLC), gas chromatography, partition chromatography, high performance (pressure) liquid chromatography (HPLC), mass spectrometry and other spectroscopic methods. Some of the more time consuming chromatographic methods may become indispensable for the isolation of pure compounds in preparative scale. Isolation is necessary for structure determination of new compounds and for procuring the necessary set of referance samples for identification of "spots" or "retention times" in chromatographic methods.

In the following we shall discuss the potential taxonomic value of chemical analysis for phloroglucinols in the fern genus *Dryopteris* Adans. (fam. *Dryopteridaceae).*

2. **The phloroglucinols**. Nearly all members of the fern genus *Dryopteris* contain in their rhizomes and stipe bases some phenolic compounds known as phloroglucinol-derivatives or "acyl-phloroglucinols", located in typical internal glands (secreting hairs) see review in Huurre et al. (1979). These possess strong anthelmintic properties and the powdered rhizome (Rhizoma filicis or Radix Filicis maris) or crude extracts (Extractum filicis aetherum), particulary of *D. filix-mas* (L.) Schott (see A. Tschirch, O. Oesterle 1900: 241-246 and H. Zörnig 1909 I:512) have been used as a cure against tape worm since antiquity. Although their use is dangerous owing to high general toxicity and is regarded as outmoded, the pronunced activity is the reason that their chemistry has been studied extensively since the 19th century and is well known today. This same knowledge makes it possible to use chemical analysis as an aid for solving some taxonomic problems associated with *Dryopteris*. We shall describe some pratical examples but only after giving a short review of chemical structures, biosynthesis and analytical methods. These are described in many articles and by different authors. For the nonspecialist it is difficult to find how best to proceed in practical analyses. In this review article we shall try to give good advice and some suggestion for improved methods.

2.2. **Chemical structure.** Formulae 2-26 give the structures of the majority of phloroglucinols so far isolated from different *Dryopteris* species, i.e. those compounds for which the structures are elucidated (see Berti & Bottari 1968; Penttilä & Sundman 1970; Widén et al. 1971, 1973, 1975, 1976 and literature cited therein). In *Arachniodes* two more compounds, iso-aspidin- AB and - BB have been detected by TLC. Apart from these compounds often called "filicins" or "aspidins" some other phloroglucinol derivatives such as flavanoids, ceroptens etc. (see Berti & Bottari 1968, Tanaka et al. 1979) occur in ferns but will not be discussed here. We shall deal only with the compounds most typical for *Dryopteris*, produced in special organs (internal glands in the rhizomes).

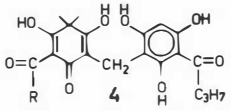
The formulae show that the different *Dryopteris* species produce a great many such compounds. Formally they are all derivatives of phloroglucinol (*1*) which, however, has never been found in ferns and does not represent a precursor in the biosynthesis of the true fern compounds (see below). The latter are, nevertheless, closely related to each other, containing similar building units (substituted hexacyclic rings), clearly due to analogous biosynthesis (see below). They can roughly be classified according to the number of hexacyclic rings. So far only two 1-ring compounds (*2* and *3*) have been observed: aspidinol (*2*), and fraginol (*3*). Aspidinol (*2*) is probably always an artefact, produced by cleavage of sensitive compounds [particularly para-aspidin (*7*), trispara-aspidin (*20*), margaspidin (*13*), etc.] during the analytical work or during prolonged (many years) storage of plant material. Fraginol (*3*), only recently isolated from *D. fragrans*, is a typical compound of this species. Most of the natural phloroglucinols contain 2, 3 or 4 hexacyclic rings bound together by a methylene bridge.



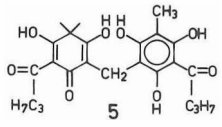
1 Phloroglucinol

2 Aspidinol - B

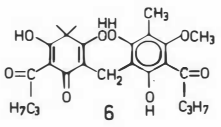
3 Fraginol - B



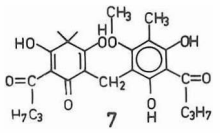
4 Norflavaspidic acid
(R=CH_3)=AB; (R=C_3H_7)=BB



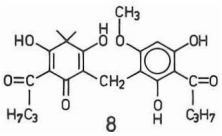
5 Flavaspidic acid - BB



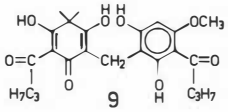
6 Aspidin - BB



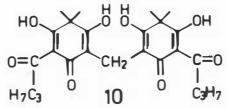
7 Para - aspidin - BB



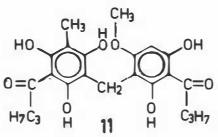
8 Desaspidin - BB



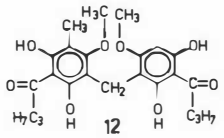
9 Orthodesaspidin - BB



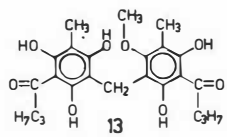
10 Albaspidin - BB



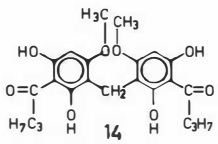
11 Phloraspin - BB



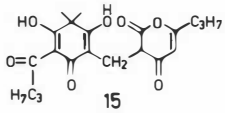
12 Phloraspidinol - BB



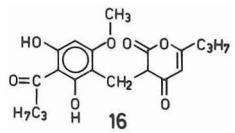
13 Margaspidin - BB



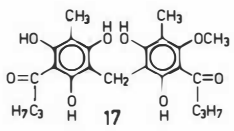
14 Methylene - bis - desaspidinol - BB



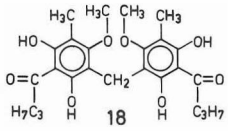
15 Phloropyron - BB



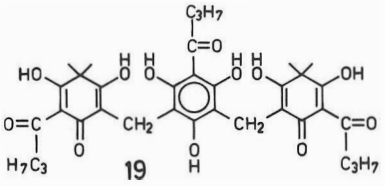
16 Phloraspyron - BB



17 Aemulin - BB

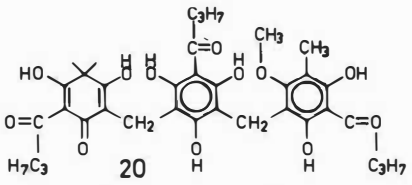


18 Methylene - bis - aspidinol - BB



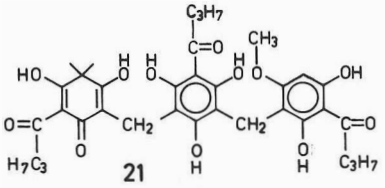
19

Filixic acid - BBB



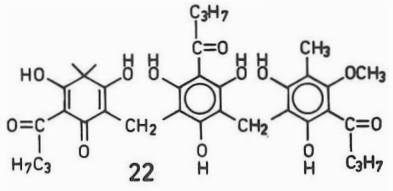
20

Trispara-aspidin-BBB



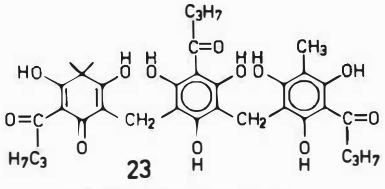
21

Trisdesaspidin-BBB



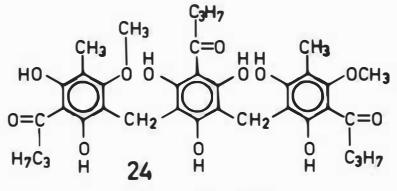
22

Trisaspidin-BBB



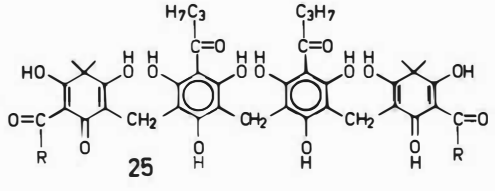
23

Trisflavaspidic acid - BBB



24

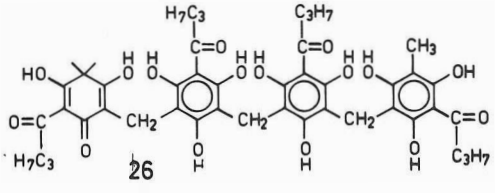
Trisaemulin - BBB



25

ABBA(R=CH_3)=Methylene-bis-norflavaspidic acid - ABBA = Dryocrassin

BBBB(R=C_3H_7)=Methylene-bis-norflavaspidic acid - BBBB



26

Tetraflavaspidic acid - BBBB

2 = Aspidinol-B. $C_{12}H_{16}O_4$ (224). Isolation and synthesis R. Boehm, Arch. exper. Path. Pharm. *38*:35(1897); Lieb. Ann. Chem. *318*:245(1901); *329*:269(1903). See also P. Karrer & Fr. Widmer, Helv. Chim. Acta *3*:392(1920); A. Robertson & W.F. Sandrock, J. Chem. Soc. *1933*:819. NMR-spectrum see Helv. Chim. Acta *53*:2184 (1970): mass spectrum see Lounasmaa et al. (1972).
Aspidinol-B is prohahly always an artefact produced from para-aspidin (7) and other compounds during isolation procedures, mainly by heating, alkaline reaction, contact with SiO_2 etc., but also during drying or long storage of rhizomes, see A. Penttilä & J. Sundman, Planta medica *14*:157(1966).

3 = Fraginol-B. $C_{13}H_{18}O_4$ (238). Isol. from *D. fragrans*: B.A. Krivut & L.M. Molodozhnikova, Khim. Prir. Soedin *6*:684(1970), Chem. Abst. *74*:95244X (1971); L.M. Molodozhnikova, A.I. Bankovskii, N.M. Sergeev & A.I. Shreter, Chimico-Farm. Zurnal, *5*:32(1971), Chem. Ahstr. *75*:11584X (1971). Synth. (as 5n - hutyryl - 3 - methyl filicinic acid) m.p. 87°, H. Riedl e K.H. Risse; m.p. 78-79°, M. Lounasmaa, C.-J. Widén & A. Huhtikangas, Acta Chem. Scand. B 28:1209 (1974b) incl. NMR and mass spectral data.

4-AB = Norflavaspidic acid-AB. $C_{21}H_{24}O_8$ (404). Isol. from *D. dickinsii*, m.p. 105-107°: H. Hisada, K. Shiraishi & J. Inagaka, Phytochem, *11*:2881(1972) incl. NMR spectrum.

4-AP = Norflavaspidic acid - AP. $C_{20}H_{20}O_8$ (390). Isol. from *D. atrata*, m.p. 103-105° (Widén et al. in preparation).

4-BB = Norflavaspidic acid-BB. $C_{23}H_{28}O_8$ (432). In pure state so far only synthetic m.p. 119-121°, A. Penttilä & J. Sundman, Acta Chem. Scand. *17*:2370 (1963).

5-AA = Flavaspidic acid-AA not yet isolated in pure form.

5-AP = Flavaspidic acid AP. $C_{21}H_{24}O_8$ (404). Isol. from *D. goldiana*, m.p. 102-106° see footnote in Lounasmaa et al. (1972:90), incl. mass spectrum.

5-AB = Flavaspidic acid-AB. $C_{22}H_{26}O_8$ (418). Isol. from *D. oreades* (= *D. abbreviata*), m.p. 209-213°, C.-J. Widén, J. v. Euw & T. Reichstein, Helv. Chim. Acta *54*:2824 (1971) with NMR spectrum p. 2831; from *D. polylepis*, m.p. 203-205°; S. Hisada, K. Shiraishi & I. Inagaki, Phytochem. *10*:2541 (1971); Yakugaku Zasshi *92*:284 (1972) with NMR spectrum p. 285; from *D. sieboldii*, m.p. 205-207°, S. Hisada, O. Inoue & I. Inagaki, Phytochem. *12*:1492 (1973). Synth. m.p. 210-212°, A. Penttilä & J. Sundman, Acta Chem. Scand. *18*:344 (1964). Mass spectrum Lounasmaa et al. (1972).

5-PB = Flavaspidic acid-PB. $C_{23}H_{28}O_8$ (432). Isol. from *D. sieboldii*, m.p. 154-156°, S. Hisada, O. Inoue & I. Inagaki, Phytochem. *12*:1494 (1973). Synth. m.p. 170-171°, Penttilä & Sundman (1964).

5-BB = Flavaspidic acid-BB. $C_{24}H_{30}O_8$ (446). = Polystichocitrin of Pohlsson (1895, 1898, Arch. exp. Pathol. Pharmak, *35*: 97-104; *41*: 246-264) Isol. from "Extractum filicis aethereum". R. Boehm, Arch. exp. Path. Pharm. *38*:35 (1897); Lieb. Ann. Chem. *318*:253 (1901); *318*;277 (1901); *329*:310 (1903); from *D. filix-mas* and *"D. austriaca"* J. Maizite, Arch. Pharm. *280*:173 (1942); A. Aehi, J. Büchi & A. Kapoor, Helv. Chim. Acta *40*:266 (1957); *D. tyrrhena* Fraser-Jenkins et Reichst. (= *"D. filix-mas var. rigidiformis"*) C. J. Widén et al. 1971; *D. polylepis*, C.J. Widén et al. (1975); *D. chrysocoma*, H.S. Puri et al. (1976) and other species. Two forms are known. From methanol a-form, m.p. 94°, from henzene B-form, m.p. 156-157°, showing similar x-ray diffraction, O. Erämetsä & A. Penttilä, Acta Chem. Scand. *24*:3335 (1970). NMR spectrum Helv. Chim. Acta *54*:2830 (1971). Synth A. McGookin, A. Rohertson & T. H. Simpson, J. Chem. Soc. (London) *1953*:1828; W. Riedl, Lieb. Ann. Chem. *585*:32 (1954).

6-AA = Aspidin-AA. $C_{21}H_{24}O_4$ (404). Isol. from *D. gymnosora* (Hisada et al. 1974), m.p. 135-136°.

6-AB = Aspidin-AB. $C_{23}H_{28}O_4$ (432). Isol. from *D. dilatata* (Widén 1967a) and *D. intermedia*, C.-J. Widén, Ann. Acad. Sci fenn. A. IV Biologia 143:1-19 (1969), m.p. 118-120°, mass spectrum Lounasmaa et al. (1971); NMR spectrum Hidén et al. (1976). Also from *D. aemula*, *D. azorica* and other species, Widén et al., Helv. Chim. Acta *58*:880 (1975a).

6-BB = Aspidin-BB. $C_{25}H_{32}O_4$ (460). = Polystichin of Pohlsson (1895, 1898) Isol. from "Extractum filices aethereum" R. Boehm (1897, 1903); from *D. carthusiana* (= *D. spinulosa*) and synth. W. Riedl & R. Mitteldorf, Chem. Ber. *89*:2595 (1956), m.p. 124-125°. Isol. also from *D. "austriaca"*, A. Aebi et al. (1957), A. Penttilä & J. Sundman (1961), from *D. intermedia*, Widén (1969), from *D. gymnosora*, S. Hisada, O. Inoue & I.

Inagaki, Phytochem. *13*:655 (1974) and other species. Mass spectrum Lounasmaa et al. (1972), NMR spectrum Helv. Chim. Acta *59*:1737 (1976).

7-AB = Para-aspidin-AB. $C_{23}H_{28}O_8$ (432). Not isolated in pure state, synth. Britton & Widén (1974), m.p. 137-140°, m/e 432.

7-PB = Para-aspidin-PB. $C_{24}H_{30}O_8$ (446). Not isolated in pure state, synth. Britton & Widén (1974), m.p. 120-22°, m/e 446.

7-BB = Para-aspidin-BB. $C_{25}H_{32}O_8$ (460). Most likely = Flavopannin of A. Heffter (1897) see Widén et al. (1973b: 2136, footmole 30). Isol. from *D. "austriaca"*, and synth., m.p. 123-125°; A. Penttilä & J. Sundman, Acta Chem. Scand. *16*:1251 (1962). From *D. remota*; C.J. Widén et al. Helv. Chim. Acta *53*:2176 (1970) with NMR spectrum; mass spectrum, Lounasmaa et al. 1972. Isolated also from other species.

8-AB = Desaspidin-AB. $C_{22}H_{26}O_8$ (418). Not isolated in pure state. Synth. Penttilä & Sundman, Acta Chem. Scand. *18*:344 (1964), m.p. 145-147°; m/e 418, Britton & Widén (1974).

8-PB = Desaspidin-PB. $C_{23}H_{28}O_8$ (432). Synth. Penttilä & Sundman (1964), m.p. 141-142°.

8-BB = Desaspidin-BB. $C_{24}H_{30}O_8$ (446). Isol. from *D. "austriaca"*, m.p. 150,5°: A. Aebi, J. Büchi & A. Kapoor, Helv. Chim. Acta *40*:266 (1957); m.p. 152-154°. A. Penttilä & J. Sundman, J. Pharm. Pharmacol. *13*:531 (1961). Also from *D. villarii*, m.p. 150-152°, C.J. Widén et al., Helv. Chim. Acta *54*:2824 (1971) and from other species. NMR spectrum, Helv. Chim. Acta *56*:836 (1973). Mass spectrum, Lounasmaa et al. (1972) and Lounasmaa (1973).

9-AB = Orthodesaspidin-AB. $C_{22}H_{26}O_8$ (418). Not isolated pure but synth. Widén et al. (1975), m.p. 149-150°, m/e 418.

9-BB = Orthodesaspidin-BB. $C_{24}H_{30}O_8$ (446). Isol. from *D. "austriaca"*, m.p. 131-133°, synth. m.p. 133-135°: A. Penttilä & J. Sundman, Acta Chem. Scand. *18*:1292 (1964).

10-AA = Albaspidin-AA. $C_{21}H_{24}O_8$ (404). Isol from *D. patula,* m.p. 162-164°, R. Tryon et al., Phytochem. *12:* 683 (1973). incl. NMR and mass spectral data. NMR see also Hisada et al. (1972c). Synth. Penttilä & Sundman (1964), m.p. 170-171°.

10-AB = Albaspidin-AB. $C_{23}H_{28}O_8$ (432). Isolated from *D. clintonigra,* m.p. 134-136° (not pure), Widén & Britton (1971c).

10-AP = Albaspidin-AP. $C_{22}H_{26}O_8$ (418). Isol. from *D. goldiana,* m.p. 149-151° (not pure), Widén & Britton (1971c).

10-PP = Albaspidin-PP, not isolated in pure state, synth. m.p. 135-137°, Penttilä & Sundman (1964a).

10-BB = Albaspidin-BB. $C_{25}H_{32}O_8$ (460). Isol. from *D. filix-mas*, m.p. 150° and synth. W. Riedl (1954); from *D. "austriaca"*, m.p. 146-147°, A. Aebi et al. (1957a); m.p. 153-154° A. Penttilä & J. Sundman (1964a); also from *D. expansa* (= *D. assimilis*) C.-J. Widén (1969) and *D. championii* Widén et al. (1975). NMR spectrum Helv. Chim. Acta *56*:836 (1973); mass spectrum Lounasmaa et al. (1972). Synth. R. Boehm, (1901c); E. Aho, Ann. Univ. Turkuensis (Finland) Ser. A I, *29*: 1-123 (1958).

11-BB = Phloraspin-BB. $C_{23}H_{28}O_8$ (432). Isol. from commercial extracts, m.p. 211°: R. Boehm, Lieb. Ann. Chem. *329*:338 (1903); from *D. "austriaca"*, m.p. 211-212° and synth. m.p. 212-213°: A. Penttilä & J. Sundman, (1961c). Also isol from *D. marginalis*, C.-J. Widén & D.M. Britton (1971d) and other species, Widén et al. (1975a), including NMR spectrum mass spectrum Lounasmaa et al. (1972).

12-BB = Phloraspidinol-BB. $C_{24}H_{30}O_8$ (446). Isol. from *D. "austriaca"* and synth. m.p. 193-194°: A. Penttilä & J. Sundman (1963b). Also isolated from *D. marginalis*, C.-J. Widén & D.M. Britton (1971d), *D. aemula* and other species, Widén et al. (1975a) including NMR spectrum; mass spectrum Lounasmaa et al. (1972).

13-AB = Margaspidin-AB not yet isolated in pure state.

13-BB = Margaspidin-BB. $C_{24}H_{30}O_8$ (446). Isol. from *D. marginalis* and synth., m.p. 178-180°: A. Penttilä & G.J. Kapadia, J. Pharm. Sci *54*:1362 (1965); m.p. 174-176°: C.-J. Widén & D.M. Britton (1971d); from *D. bissetiana*, S. Hisada, S. Yasuno & J. Inagaki, Yakugaku Zasshi *91*:687 (1971) with NMR spectrum, for this see also Helv. Chim. Acta *58*:894 (1975) and isolation from *D. aemula*.

14-BB = Methylen-bis-desaspidinol. $C_{23}H_{28}O_8$ (432). Present in *D. "austriaca"*, synth. m.p. 174-175°. A. Penttilä & J. Sundman (1963b); from *D. marginalis* and synth. m.p. 176-179°: C.-J. Widén & D.M. Britton (1971d). Mass spectrum Lounasmaa et al. 1972; Helv. Chim. Acta *56*:1141 (1973).

15-BB = Phloropyron-BB. $C_{21}H_{26}O_7$ (390). Isol. from *D. "austriaca"* and synth., m.p. 111-112°, A. Penttilä & J. Sundman (1961a); J. Pharm. Pharmac. *13*:531 (1961); from *D. expansa* C.-J. Widén et al. (1970); from *D. expansa* (N-America) and *D. campyloptera*: C.-J. Widén & D. M. Britton (1971a). Mass spectrum Lounasmaa et al. (1972), NMR spectrum Helv. Chim. Acta *59*:1738 (1976)

16-BB = Phloraspyron-BB. $C_{20}H_{24}O_7$ (376). Isol. from *D. "austriaca"* and synth. m.p. 135-136°: A. Penttilä & J. Sundman (1963b).

17-AB = Aemulin-AB not yet described in pure state.

17-BB = Aemulin-BB. $C_{24}H_{30}O_8$ (446). Isol. from *D. aemula*, m.p. 90-91°: C.-J. Widén M. Lounasmaa, G. Vida et T. Reichstein, Helv. Chim. Acta *58*:880 (1974). NMR spectrum and mass spectrum ibid.

18-BB = Methylen-bis-aspidinol-BB. $C_{25}H_{32}O_8$ (460). Isol. from *D. marginalis*: C.-J. Widén & D.M. Britton (1971d). Synth. R. Boehm (1903a: 286), m.p. 190-191°, improved method in acetic acid: E. Aho, Ann. Univ. Turkuensis (Finland), Ser. A I. *29*:106 (1958), see also W. Riedl & R. Mitteldorf (1956b). NMR spectrum Helv. Chim. Acta *58*:893 (1975), mass spectrum Helv. Chim. Acta *56*:1141 (1973).

19-ABA = Filixic acid-ABA. $C_{32}H_{36}O_{12}$ (612). Isol. from *D. dickinsii* and synth. m.p. 164-167°: S. Hisada, K. Shiraishi & I. Inagaki (1972b, c, d). From *D. parallelogramma*, m.p. 161-162°: R. Tryon, C.-J. Widén, A. Huhtikangas & M. Lounasmaa, Phytochem. *12*:683 (1973). NMR spectrum Yakugaku Zasshi *92*:1125 (1972), mass spectrum Tryon et al. (1973) (Main peaks are given).

19-ABP = Filixic acid-ABP. Present in *D. filix-mas* and other species: A. Penttilä & J. Sundman (1963a), not isolated in pure state.

19-ABB = Filixic acid-ABB (as the former one).

19-PBP = Filixic acid-PBP. $C_{34}H_{40}O_{12}$ (640). Isol. from *D. filix-mas* and synth. m.p. 192-194°, A. Penttilä & J. Sundman (1963a).

19-PBB = Filixic acid-PBB. $C_{35}H_{42}O_{12}$ (654). Isol. from *D. filix-mas* m.p. 184-186°: A. Penttilä & J. Sundman, Acta Chem. Scand. *17*:191 (1963).

19-BBB = Filixic acid-BBB. $C_{36}H_{44}O_{12}$ (668). Isol. from *D. filix-mas* (as mixture of homologues): E. Luck, Lieb. Ann. Chem. *54*:119 (1845); Ber. Deutsch. Chem. Ges. *21*:3465 (1888), m.p. 184.5° and many others. Pure from *D. "austriaca"* and synth. m.p. 172-174°: A. Penttilä & J. Sundman, (1963a, c); from *D. villarii*, m.p. 170-172°: C.-J. Widén, et al. (1971a). NMR spectrum Helv. Chim. Acta *56*:837 (1973), mass spectrum Helv. Chim. Acta *54*:2855 (1971); Planta med. *24*:154 (1973); Helv. Chim; Acta *56*:1139 (1973).

20-BBB = Trispara-aspidin-BBB. $C_{36}H_{44}O_{12}$ (668) Isol. from *D. remota*, m.p. 143-147° or 157-160°: C.-J. Widén, J. v. Euw & T. Reichstein, Helv. Chim. Acta *53*:2176 (1970). Also isol. from *D. inaequalis* and *D. sacrosancta* [erroneously first assumed to be "trisaspidinol", see Helv. Chim. Acta *59*:1725 (1976)]. NMR spectrum Helv. Chim. Acta *53*:2185 (1970) and *56*:1143 (1973) sub "trisaspidinol"; mass spectrum Helv. Chim. Acta *54*:2856 (1971).

21-BBB = Trisdesaspidin-BBB. $C_{35}H_{42}O_{12}$ (654). Isol. from *D. "austriaca"*, m.p. 148-152° (decomp.) and synth. m.p. 142-146° (decomp.): A. Penttilä & J. Sundman (1963c). NMR spectrum Helv. Chim. Acta *53*:2185 (1970), mass spectrum Helv. Chim. Acta *54*:2856 (1971).

22-BBB = Trisaspidin-BBB. $C_{36}H_{44}O_{12}$ (668). Isol. from *D. "austriaca"*, m.p. 156-159° and synth. m.p. 155-158°: A. Penttilä & J. Sundman (1963c).

23-BBB = Trisflavaspidic acid-BBB. $C_{35}H_{42}O_{12}$ (654). Isol. from *D. "austriaca"*, m.p. 169-174° (decomp.) and synth. m.p. 168-174° (decomp.): A. Penttilä & J. Sundman (1963c). From *D. aitoniana* m.p. 168-174° : C.-J. Widén, G. Vida, J. v. Euw & T. Reichstein (1971a). NMR spectrum not publ., mass spectrum, Helv. Chim. Acta *54*:2856 (1971).

24-BAB = Trisaemulin-BAB. $C_{34}H_{40}O_{12}$ (640). Isol. from *D. aemula*, m.p. 180-183°: C.-J. Widén et al. (1975a). NMR spectrum idem 897, mass spectrum idem 892.

24-BBB = Trisaemulin-BBB. $C_{36}H_{44}O_{12}$ (668). Isol. from *D. aemula*, m.p. 168-170°: C.-J. Widén et al. as above. Mass spectrum Helv. Chim. Acta *58*:892 (1975).

25-ABBA = *Menthylen-bis-norflavaspidic acid-ABBA* (= Dryocrassin). Isol. from *D. crassirhizoma*, m.p. 209-214°: Y. Noro et al. (1973); m.p. 210-215°: C.-J. Widén et al. (1975d). NMR data Y. Noro et al. (1973) and C.-J. Widén et al. (1975 d). Mass spectrum Acta Chem. Scand. *B 29*:859 (1975).

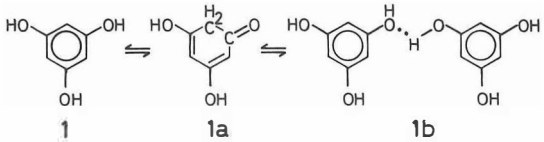
25-BBBB = Methylen-bis-norflavaspidic acid-BBBB. $C_{47}H_{56}O_{16}$ (876). Isol. from *D. "austriaca"* and synth. m.p. 158-165°: A. Penttilä & J. Sundman (1973c).

26-BBBB = Tetraflavaspidic acid-BBBB. Isol. from *D. filix-mas* and *D. aitoniana* m.p. 180-183°, J. v. Euw. et al. (Publ. in preparation).

In reality the situation is still more complicated. In most of the given formulae only the compounds with butyryl side chains $CH_3—CH_2—CH_2—CO—$ (abbreviated as B-derivations) are represented. Very often these compounds are accompanied by lower and rarely by higher homologues, carrying shorter, i.e. $CH_3—CH_2—CO—$ (= acetely) and occasionally longer $CH_3—CH_2—CH_2—CH_2—CO$ (= valeryl) side chains, e.g. in *D. schimperana [Widén et al. 1973: 1133, 2125]*. These are abbreviated as A-, P- and V- derivatives respectively. In ferns branched chains, e.g. $(CH_3)_2—CH—CO—$ (= isobutyryl) have so far been observed only in *D. erythrosa* [Widén et al. 1975c] but are known to occur in *Hagenia abyssinica* [Lounasmaa et al. 1973, 1974 and references therein].

In some cases, e.g. norflavaspidic acid *4*, not only the BB compound (formula *4-BB*) is known in pure state, but also the AB compound (formula 4-AB). In other cases e.g. flavaspidic acid (*5*) in which only the BB derivative is formulated, many more such homologues are known in reasonably pure state and are mentioned in the bibliography to formulae. Very often the separation of such homologues is difficult and has not been accomplished, the presence of lower or higher homologues in the mixture being established by degradation or by spectroscopic methods such as mass spectrometry (MS) and nuclear magnetic resonance (NMR).

Phloroglucinol (1) can also exist in a tautomeric form (1a) and both 1 and *1a* may be present in equilibrium in solution.
They can also form

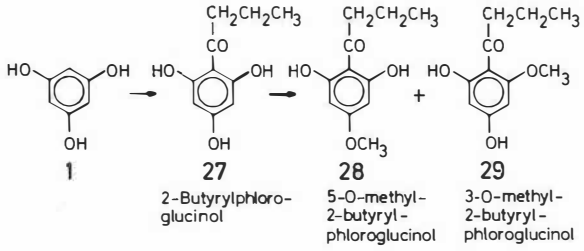


1 1a 1b

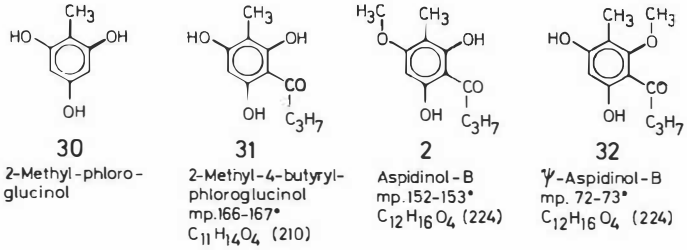
dimers *(1b)* and polymers by hydrogen bonding. This is true for all the mentioned fern constituents. In each of the formulae *2-26* only one of these possible isomers (most probably the dominant end form of monomers) is given. NMR spectra show that an equilibrium with other forms, and probably different associates as well, often present in solution [see Widén et al. 1976].

The structure of these compounds was elucidated by degradation and partly by synthesis, confirmed by physical methods, particularly mass spectra and NMR.

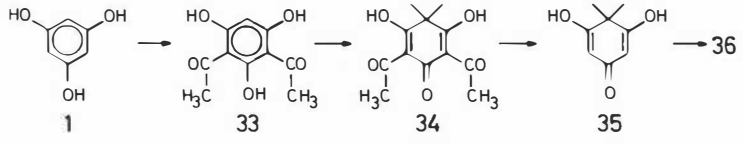
2.3 **In vitro synthesis** of the monocyclic units 27, 28 and 29



and homologues can easily be accomplished by acetylation and partial O-methylation of phloroglucinol (1) (reviewed in Widén et al. 1973). The methyl derivatives 31, 2 and 32 are less readily available, because methyl-phloroglucinol (30) is no longer commercially obtainable. The important butyryl dilicinic acid (36) can best be

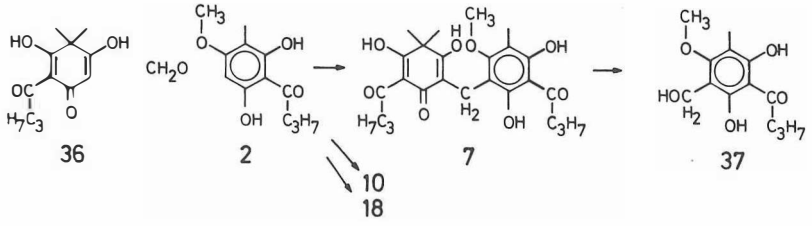


obtained by mild reductive cleavage (see below) of natural phloroglucinols [Boehm 1901a, 1903a] or by C-acetylation of filicinic acid (*35*) [Riedl & Risse 1954b; Anderssen 1968a; Widén 1968], which can be synthesised from phloroglucinol (*1*) via *33* and *34*. [Hoefer et Riedl 1962]. It is also possible to hydrolise only one acyl side chain in *34* yielding acetyl filicinic acid (*36a*). In this way *36* or homologues can be prepared in good yields [Andersen et al. 1968b; Meikle & Stevens 1978].

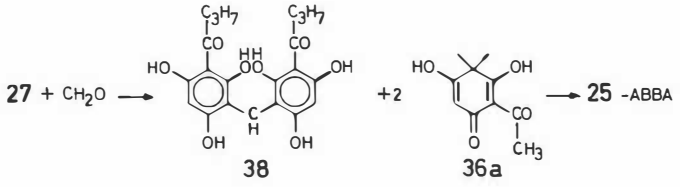


The 2-, 3- and 4-ring compounds can in principle be obtained from the monocyclic units by "methylenation", usually performed with formaldehyde in slightly alkaline solution [Boehm 1903a; Riedl 1954], and sometimes better in acetic acid [Aho 1958:106]. We describe here only the synthesis of para-aspidin (7) from *36* and *2* as an example [Penttilä & Sundman 1962:1251] of a 2-ring compound. In this case the asymetrical compound 7 is the major product, but the asymetrical

ones *10* and *18* are also formed and in many other cases a separation of the mixture becomes unavoidable. The reaction is reversible and sensitive benzyl alcohols *37* or their equivalents are formed as intermediates.



Difficulties increase in the attempt to make 3-ring compounds, but the filixic acid-BBB (*19*) and some symmetrical homologues are easily obtained from *36* (or homologues) and *27* with formaldehyde [Penttilä & Sundman 1963:191]. Only two 4-ring compounds (*25* and *26*) have so far been found in ferns. For *25* two homologues (*25*- ABBA and *25*-BBB) have been observed, both symmetrical and both synthesised in two different ways. The intermediate *38* (obtainable from *27* with formaldehyde) was condensed with formaldehyde and two



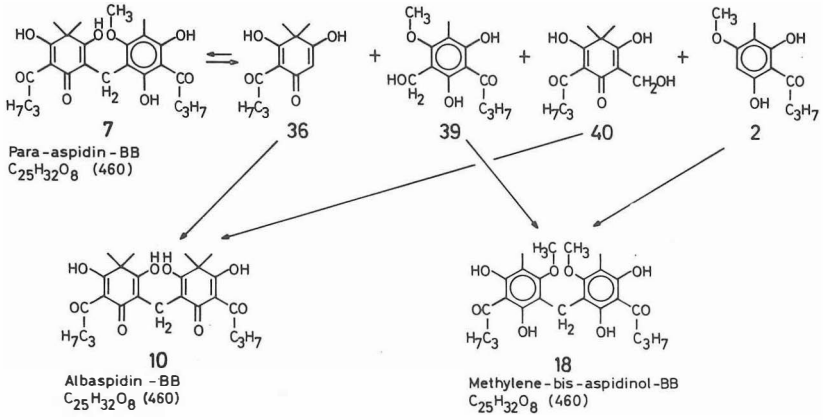
molecules of acetylfilicinic acid (*36a*) to give *25*-ABBA [Noro et al. 1973], while the *25*-BBB homologue was prepared from norflavaspidic acid (*4*-BB) and formaldehyde [Penttilä & Sundman 1963:2370].

2.4 **Rottlerone rearrangement.** The above mentioned reversal of the "methylenation can lead in some phloroglucinols to marked disproportion (scheme a) known as "rottlerone rearrangement". This is typical for asymmetric polydroxy diacyl diphenylmethanes

Scheme a: 

when x and y are acyl resorcinol- or acyl phloroglucinol-residues. The reaction proceeds best by warming in aqueous acetic acid but also in alkaline solution [McGookin et al. 1951, 1953]. These authors showed that hydrolytic cleavage

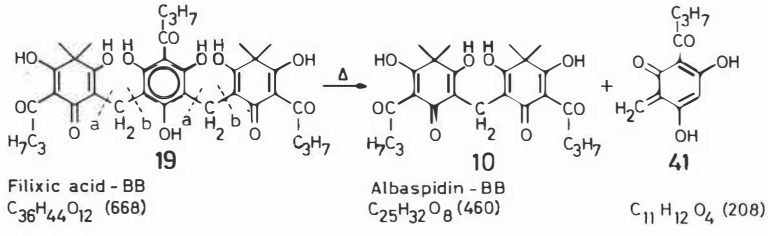
occurs at the methylene bridge in both possible ways (a and b) giving four monocyclic intermediates. Para-aspidin-BB (7) for example would yield *36*, *39*, *40* and *2*. Recombination of *36*+*39* would give the asymmetrical



Scheme b: Example of rottlerone rearrangement in para-aspidin-BB.

para-aspidine (7) back. But the other combinations, *36*+*40* and *39*+*2*, will produce albaspidine (*6*) and methylene-bis-aspidinol (*18*), respectively. The same or equivalent products are obtained by heating [Penttilä & Sundman 1963; Penttilä 1967] and are important in interpretation of mass spectra [Lounasmaa et al. 1971, 1973; Lounasmaa 1973:152].

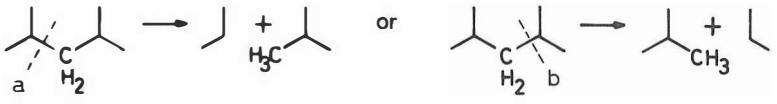
Still more puzzling in the effect of the rottlerone rearrangement in tri- and tetracyclic phloroglucinols (see Scheme c). Filixic acid (*19*) is known to produce albaspidin (*6*) upon heating [B. Widén 1944; Penttilä & Sundman 1963; Penttilä 1967] and in the mass spectrum



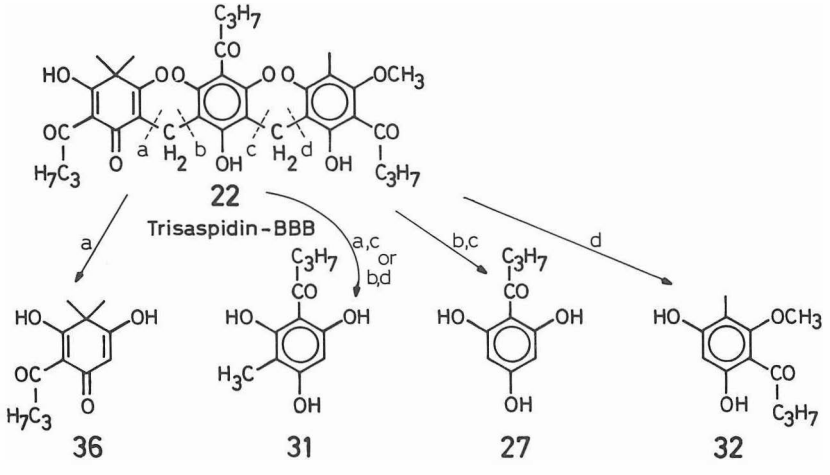
Scheme c: Example of rottlerone rearrangement in filixic acid (19).

the peak at m/e 460 may become the base peak at low electron voltage [see fig. 1 in Lounasmaa et al. 1971:2855]. Formation of the hypothetical product *41* can be deduced from the fairly strong peak at m/e 209, interpreted as protonated *41*.

2.5. **Degradation.** For structure determinations the reductive cleavage with Zn dust in aqueous 5 % NaOH solution under mild conditions (5 minutes warming at 100°) [Boehm 1901, 1903] is the most important reaction. Penttilä & Sundman [1961, 1962, 1963a, b, 1964, 1967, 1970, see also Widén et al. 1967, 1969, 1973] have used this method with great success in their extensive studies on different fern phloroglucinols. We quote here their result for trisaspidin-BBB (*22*). The molecule is broken again at the methylen bridge which is transformed into a methyl group, and this again can happen in two ways (a and b).



Theoretically, then, trisaspidin (*22*) can yield seven different monomers. The mentioned authors could detect four of them (*36*, 31, 27 and 2) see scheme d.



Scheme d. Monomers obtained by reductive cleavage of trisaspidin (*22*).

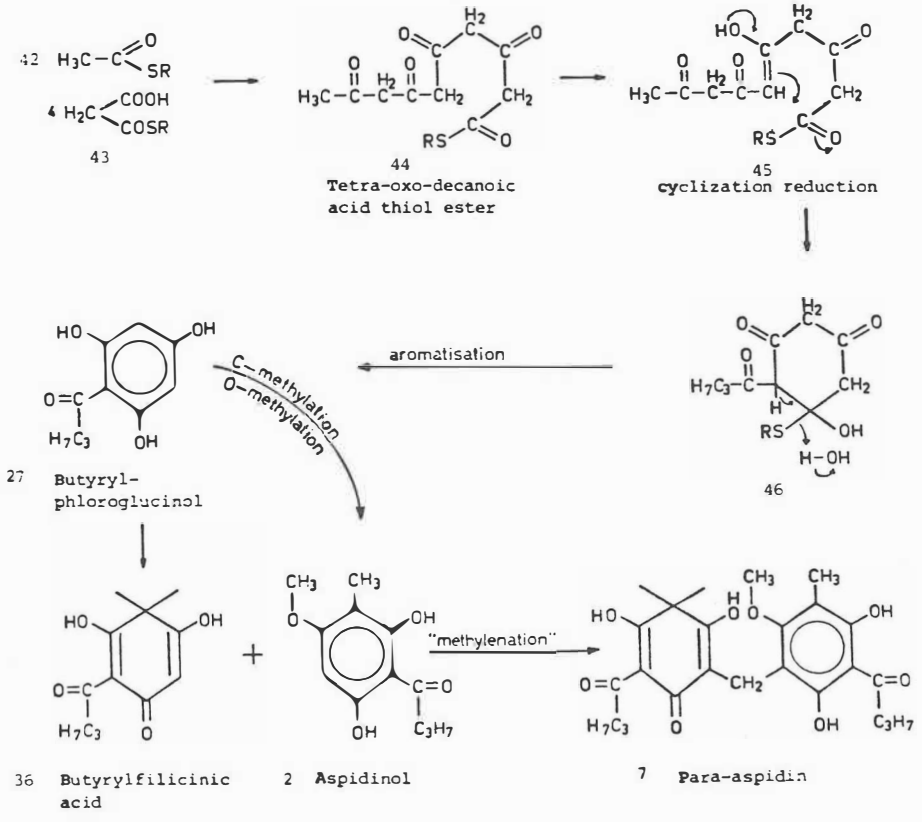
Detection of such clevage products is best performed with a combination of PC and TLC. Widén et al. [1973] have recently reviewed the methods and given RF values for all homologues (A, P, B, V) of acylfilicinic acids, aspidinols, acylphloroglucinols and acyl-methylphloroglucinols. Widén et al. [1975:889] gave also such values for desaspidinols and isomers of aspidinol B (i.e. iso-aspidinol and -aspidinol (*32*).

If sufficient material is not available for identification of such hexacyclic cleavage products or if sufficient time cannot be devoted to the work, a more vigorous method can be used for a semiquantitative overall estimation of homologues. Reductive cleavage performed under such harsh conditions (24 hrs heating with Zn dust in 15 % NaOH at 100°) splits the acyl residues off carbonic acids, which then are identified and semiquantitatively estimated by gas chromatography [Widén et al. 1973:2141-2142].

2.6. **Biosynthesis.** Scheme e shows the biosynthesis of fern phloroglucinols as postulated by Penttilä (1967, see also Berti and Bottary 1968, Geissman 1967). She verified most of the important steps by using radioactive markers. The starting point is the coenzyme A derivative *42* of acetic acid, which is partly carboxylated to the malonic acid derivative *43*. Condensation of one molecule of *42* with four of *43* yields the tetra-oxo-decanoic acid derivative *44* which after cyclization and side chain reduction gives butyryl-phloroglucinol *27*. This is the key compound, from which all other fern phloroglucinols are formed. In this simplified scheme the formation of para-aspidin (*7*) is formulated as an example. For this purpose one molecule of butyryl-phloroglucinol (*27*) is transformed into butyryl-filicinic acid (*36*) by geminal dimenthylation and a second mol of *27* into aspidinol (*2*) by C- and O-methylation. The two components (*36* and *2*) must now be bound together by "methylenation". As mentioned earlier this can be performed in the laboratory with formaldehyde. Nature makes para-aspidin in two steps, by C-methylation and subsequent oxydative condensation. Formation of 3- and 4-ring compounds takes place when one or two units of butyryl-phloroglucinol (*27*) are inserted by "methylenation" between the two other hexacyclic rings.

If in the formation of the polyketone only 3 units of the malonic acid (*43*) participate, a tri-oxo-octanoic acid derivative (lower homologue of *44*) is formed, which further leads to the A-homologues (carrying acetyl groups). If propionic acid (instead of acetic acid) functions as starter, a tri-oxo-nonanoic acid derivative is formed leading to the P-homologues. According to recent results [Huhtikangas et al. 1978] some of the aliphatic side chains may be built by C-methylation from lower homologues.

The general principles of biosynthesis are the obvious reason why all natural fern phloroglucinols, in spite of their great diversity, possess analogous architecture.



Scheme e. Biosynthesis of phloroglucinols as postulated by Penttilä 1967.

3. **Practical results.** These can be summarized in four "rules" with a few exceptions) as follows:

3.1. Phloroglucinols are present in the rhizomes and thickened stipe bases in most species of the genus *Dryopteris.*

Exceptions (with no or only traces of phloroglucinols) are some forms of *D. expans* (Presl) Fraser-Jenkins & Jermy (= *D. assimilis* Walker) [Widén et al. see Tab. 2], some Japanese species: *D. sparsa* (Kam.) O. Ktze, *D. polita* Ros. and *D.hayatae* Tagawa [see Hisada & Noro 1961; Widén et al. 1976b; 1978a] and the African *D. kilemensis* (Kuhn) O. Ktze [Widén et al. 1973]. Hisada & Noro [1961] and Widén et al. [1978a] also mention *D. shikokiana* (Makino) C. Chr and *D. hendersonii*

(Bedd.) C.Chr. as devoid of phloroglucinols. These last two species however, have been transferred to *Nothoperanema* by Ching [1966]. The African *Nothoperanema squamiseta* (Hook). Ching (formerly *Dryopteris squamiseta* (Hook.) O. Ktze) likewise does not contain any phloroglucinols [Widén et al. 1973].

3.2. Outside *Dryopteris* such compounds were found only in a few closely related fern genera: *Ctenitis* [Mehra & Mittal 1961, Widén et al. 1978a; Widén & Puri 1979], *Polystichum* (so far only in *P. tsus-simense* (Hook.) J. Sm. and P. *rigens* Tagawa [Widén et al. 1976b, 1978a], in most species of *Arachniodes and in Acrophorum nodosus* [Widén et al. 1976b, 1978a]. But very similar phloroglucinols are present in some completely unrelated phanerogams: the "Kosins" in female flowers of *Hagenia abyssinica* Gmel. (Rosaceae) [see Hems & Todd 1937; Birch & Todd 1952; Riedl 1956; Orth & Riedl 1963, Lounasmaa et al. 1973; 1974a, b, Lounasmaa & Varenne 1978], the rottlerin (*do* mallotoxin) in *Mallotus philippinensis* Müll. (Euphorbiaceae) [see literature in Lounasmaa et al. 1975], the "uliginosis" in *Hypericum uliginosum* H.B.K. Hypericaceae [Parker & Johnson 1968, Meikle & Stevens 1972, 1978] and the myrtucommulones in *Myrtus communis* L. (Myrtaceae) [Kashman et al. 1974] and in *Callistemon lanceolatus* DC (Myrtaceae) [Lounasmaa et al. 1977.

3.3. The composition of phloroglucinols is usually fairly constant for each species, little dependent on age of the plant, season of collection or origin; see *D. filix-mas* (new results) and *D. aemula* in table 2 as examples. Exceptions (see also table 2) are *D. expansa* (Presl) Fraser-Jenkins & Jermy (=*D. assimilis* Walker) and *D. carthusiana* (Vill.) H.P. Fuchs. In the former there are sometimes only traces of phloroglucinols, sometimes none at all [Widén & Sorsa 1969; Widén, Sorsa & Sarvela 1970; Widén & Britton 1969; 1971:247; Widén 1972; Britton & Widén 1974:627], and in the latter some specimens are devoid of para-aspidin (*7*). This was noticed both for plants growing in Europe [Widén et al. 1970] and for plants in N-America [Widén & Britton 1971? Britton & Widén 1974:627]. *D. cristata* A. Gray sometimes contains and sometimes lacks para-aspidin [Widén & Britton 1971:1141].

3.4. Hybridis and allopolyploid species often show an additive composition in phloroglucinols when compared with their parents or ancestors [Widén & Britton 1971:1141; Widén et al. 1975b; Widén, Widén & Gibby 1978 and others]. Table 3 gives an example of such behaviour. *D. x pseudo-abbreviata* Jermy is a sterile hybrid of *D. aemula* (Aiton) O. Kuntze x *D. oreades* Fomin. Its phloroglucinols [Widén et al. 1976] show a composition that corresponds well to the arithmetic sum of the values found for the parents. This is often by no means always so; frequently one parent or ancestor will suppress formation of a component present in the other. Table 3 also gives results for *D. crispifolia* Rasbach & Reichstein [Widén et al. 1975] most probably all allotetraploid species once formed by chromosome doubling of the diploid hybrid of *D. aemula x azorica* (Christ) Alston. It contains aspidin (*6*) as major component and other phloroglucinols (*5, 7, 8, 11* and *15*) present in one or both ancestors; but phloroglucinol (*12*), margaspidin (*13*), aemulin (*17*) and trisaemulin (*24*), which are present in *D. aemula*, have not been found in *D. crispifolia*. It is

	origin	ploidy level	flavaspidic acid (5)	aspidin (6)	para-aspidin (7)	desaspidin (8)	albaspidin (10)	phloraspidinol (12)	margaspidin (13)	phloropyron (15)
D. filix-mas	He	4x	+/+++	-	+	+	-/+	-	-	-
	Br	4x	+++	-	+	+	-	-	-	-
	SF	4x	+++	-	+	+	-	-	-	-
	SF	4x	+++	-	-/++	+/++	-	-	-	-
(Eastern)	Can	4x	+++	-	+/+++	+/++	-	-	-	-
(Western)	USA	4x	+++	-	+	++	-	-	-	-
	I	(4x)	+	-	++	++	-	-	-	-
	A	(4x)	+++	-	-	+	-	-	-	-
D. aemula	Md	(2x)	(+)	++	++	(+)	-	+	+++	
	Br	(2x)	(+)	++	+	-	-	+	+++	-
	Az	(2x)	(+)	++	++	(+)	-	+	+++	(+)
	Ga	(2x)	(+)	++	++	-	-	(+)	+++	(+)
	Hb	2x	(+)	++	+	-	-	(+)	+++	-
D. expansa	He	2x	+	+++	+++	-	(+)	-	-	+++
	Br	2x	(+)/++	-/+++	+++	-	(+)/+	-	-	+++
	SF	2x	+/+++	-/+++	-/+++	(+)/+++	(+)/+	-	-	(+)/+++
	No	2x	++	-/+++	-	-	(+)	-	-	+++
	Sv	2x	++	+++	-/+++	(+)/++	+	-	-	+++
	Ru	2x	+	-/+++	-	-	+	-	-	+++
(Alaska)	USA	2x	+++	+++	+++	(+)	+	-	-	+/+++
(Eastern) (Lake superior)	USA	2x	-	-	-	-	-	-	-	-
	USA	2x	+	(+)	+++	(+)	(+)	-	-	+++
(Western)	Can	2x	+	+++	+++	-/(+)	(+)	-	-	+++
(Quebec)	Can	2x	+	(+)/++	+++	-/(+)	(+)	-	-	+++
	Can	2x	-	-	-	-	-	-	-	-
	Sib		+	-	-	+++	+	-	-	-
	Kam		+++	-/++	+	+++	(+)	-	-	-/+
	Jap	2x	+++	-/++	+	+++	(+)	-	-	-/+

Table 2 — Illustration for rule 3.3. and exceptions. Giving semiquantitative composition of main phloroglucinols (homologues not given) of three *Dryopteris* species of which two are widely distributed in the northern hemisphere. *) = New results. Abbreviations for origin: A = Austria, Az = Azores, Br = Britain, Can = Canada, Co = Corsica, Ga = France, Ge = Germany, Hb = Ireland, He = Switzerland, I = Italy, Jap = Japan, Kam = Kamtshatka, Md = Madeira, N = Norway, Sc = Scotland, Sib = Siberia, SF = Finland, Sv = Sweden, Tu = Turkey, USA = United States of America.

aemulin (17)	methylene-bis aspidinol (18)	filixic acid (19)	trispara-aspidin (20)	trisdesaspidin (21)	trisaspidin (22)	trisflavaspidic acid (23)	trisaemulin (24)	methylene-bis-norflavaspidic (25)	tetra-flavaspidic acid (26)	References
-	-	+++	-/+	-	-	(+)	-	-	(+)	Widén et al. 1971e*)
-	-	+++	-	-	-		-	-		Widén et al. 1971e
-	-	+++	-	(+)	-		-	-		Widén 1969
-	-	+++	-/+	(+)	-	(+)	-	-	(+)	Puri et al. 1976 *)
-	-	+/++	-/+	(+)	-	(+)	-	-	(+)	Widén & Britton 1971 d *)
-	-	+++	+	(+)	-	(+)			(+)	Widén & Britton 1971 d *)
-	-	+++	(+)	+	-	-	-	-	-	unpubl.
-	-	+++	-	-	-	(+)/+	-	-	(+)/+	unpubl.
++	-	-	-	-	-	-		-	-	Widén et al. 1970;a1975a
++	-	-	-	-	-	-		-	-	Widén et al. 1970a;1975a
++	(+)	-	-	-	-	-	+	-	-	Widén et al. 1975a
++	-	-	-	-	-	-	+	-	-	
++	-	-	-	-	-	-	+	-	-	Widén et al. 1976a
-	-	-	-	-	-	-	-	-	-	Widén et al. 1970a
-	-	-	-	-	-	-	-	-	-	Widén et al. 1970 a
-	-	-/+	-	-/++	-	-	-	-	-	Widén & Sorsa 1969
-	-	-	-	-	-	-	-	-	-	Widén et al. 1970 Widén et al. 1970 a
-	-	-	-	-	-	-	-	-	-	Widén et al. 1970a
-	-	-	-	-	-	-	-	-	-	Widén et al. 1970a
-	-	-	-	-	-	-	-	-	-	Widén 1969
-	-	-	-	-	-	-	-	-	-	Widén 1969
-	-	-	-	-	-	-	-	-	-	Widén & Britton 1971 a
-	-	-	-	-	-	-	-	-	-	Widén & Britton 1971 a
-	-	-	-	-	-	-	-	-	-	Britton & Widén 1974
-	-	-	-	-	-	-	-	-	-	Britton & Widén 1974
-	-	-	-	+++	-	-	-	-	-	Britton & Widén 1971 a
-	-	-	-	+++	-	-	-	-	-	Britton & Widén 1971 a
-	-	-	-	+++	-	-	-	-	-	Widén et al. 1975 c; 1978 b

known [Scora & Wagner 1964; Ehrendorfer 1969] and theorectically well understood [Farel & Ownbey 1968; Stern & Ownbey 1971; Lavie 1974] that hybrids and allopolyploids may occasionally contain new compounds ("hybrid substances") not present in either of their parents. For further literature see Widén et al. [1976:1740]. Such cases may also occur in *Dryopteris* [Widén et al. 1975b].

Two of the species, *D. filix-mas* and *D. aemula*, show relatively constant values, little dependent on origin, whereas *D. expansa* (= *D. assimilis*) shows strong individual and geographical variation. Both in N-America and Europe, some plants of *D. expansa* are poor, some even devoid of and others ± rich in phloroglucinols, and plants from Asia show a rather different composition (much less phloropyrone and more trisdesaspidin) from plants of N-America and Europe. There are also some morphological differences between plants from Eastern Asia and their Western relatives. A rather pronounced variation was also noticed in *D. carthusiana* [Widén et Sorsa 1969a, Widén 1969b, Widén et al. 1970; Widén & Britton 1971a; Britton & Widén 1974].

In table 2 and those that follow, the signs are abbreviations for the following figures: - = less than 2 %; (+) = 1-5 %; + = 5-10 %; ++ = 10-20 % and +++ = more than 20 % of the crude mixture of phloroglucinols. Some earlier values (see literature) have been corrected in this and the following table after checking the old chromatograms and comparing them with recent analytical values. Countries of origin are abbreviated as follows: A = Austria, Az = Azores, Br = Britain, Can = Canada, Co = Corsica, Ga = France, Ge = Germany, Hb = Ireland, He = Switzerland, I = Italy, Ja = Japan, Kam = Kamchatka, M = Madeira, No = Norway, Rum = Rumania, Sc = Scotland, SF = Finland, Si = Siberia, SU = Sweden, Tu= Turkey, USA = United States of America. Compounds 2, 3, 4, 9, 14 and 16 not mentioned in this table were absent in all these taxa.

In table 4 we give results for some members of the *D. dilatata* and *D. filix-mas* complexes. They show how the composition of phloroglucinols may reflect evolutionary relations, particulary in the formation of allotetraploid species.

As Walker [1955; 1961], Gibby & Walker [1977] and Gibby [1977] have shown by experimental methods, mainly cytological examination of experimental and natural hybrids, the three species known as *D. intermedia* (Muehl.) A. Gray (N-America), *D. azorica* (Azores) and *D. maderensis* (Madeira) are closely related. Cytologically they are even indistinguishable and have clearly evolved from the same ancestral stock. Today, as probably for a very long time, they are geographically widely separated. Now they are morphologically still similar but distinct [see Gibby et al. 1977, Gibby 1979] and adapted to different ecological conditions. Thus *D. intermedia* will stand severe frost in Canada, whereas *D. azorica* and *D. maderensis* are much more tender. It is interesting to see that they show a nearly identical composition of phloroglucinols.

Gibby & Walker [1977] have also solved an old problem, i.e. the origin of one of our most common wood ferns, *D. dilatata*. Using the mentioned methods they showed that it must have originated by chromosome doubling from a hybrid of one

	origin	ploidy level	flavaspidic acid(5)	aspidin(6)	para-aspidin(7)	desaspidin(8)	albaspidin(10)	phloraspin(11)	phloraspidinol(12)	margaspidin(13)	phloropyron (15)	aemulin(17)	methylene-bis-aspidinol(18)	filixic acid(19)	trisflavaspidic acid(23)	trisaemulin(24)	tetraflavaspidic acid(26)	references
D. oreades	I	2x	+++	-	-	-	-	-	-	-	-	-	-	++	+	-	+	Widén et al. 1976a*)
D. aemula	Hb	2x	(+)	++	+	-	-	-	+	+++	-	++	-	-	-	+	-	Widén et al. 1976a*)
Sum of above			++	+	+	-	-	-	(+)	++	-	+	-	+	(+)	(+)	(+)	*)
D. x pseudo-abbreviata	Sc		+	++	++	-	-	-	++	++	-	++	-	+	-		-	Widén et al. 1976a*)
D. azorica	Az	2x	+	+++	-	-	(+)	-	-	-	-	-	-	-	-	-	-	Widén et al. 1975a*)
Sum of D.aemula and of D.azorica			+	++	+	-	-	-	(+)	++	-	+	-	-		(+)	-	*)
D. crispifolia	Az	4x	+	+++	++	(+)	-	-	-	-	(+)	-	+	-	-	-	-	Widén et al. 1975a *)

Table 3 — Illustration for rule 3.4. The main phloroglucinols (homologues not given) in *D. x pseudo-abbreviata* closely correspond to the arithmetic sums of those in parents (*D. oreades* and *D. aemula*). In the allotetraploid *D. crispifolia,* formation of margaspidin (*13*) and aemulin (*17*) as well as two others minor compounds present in one ancestor (*D. aemula*) is suppressed by the other ancestor (*D. azorica*). Compounds not mentioned in the table were absent in these taxa. Abbr. see table 2.

of the three mentioned members of the *D. intermedia* group with *D. expansa*. Table 4 shows that the chemistry is in complete agreement with such an origin. Gibby [1977] further showed that the American *D. campyloptera* Clarkson contains (in principle) the same ancestral genomes ad *D. dilatata*. It is therefore quite understandable that these two species are morphologically rather similar, the small differences having arisen either during the very long time of their wide geographical separation, or from some differences in one or both of their ancestors. For geographical reasons the American *D. campyloptera* could have originated from a hybrid of *D. expansa x intermedia* and the Eurasiatic *D. dilatata from D. expansa x maderensis* (or similar taxon). Table 4 shows further that the composition of phloroglucinols in *D. campyloptera* is indeed very similar to that in *D. dilatata*, though certain differences (e.g. in content of phloropyron) do occur.

A similar agreement was observed for *D. filix-mas*. As shown by Manton [1950:44-62] and Manton & Walker [1954] *D. filix-mas* is an allotetraploid species which originated through chromosome doubling in a hybrid of *D. oreades* Fomin (= *D. abbreviata* auct.) with another diploid species not known at that time. The species has since been identified as *D. caucasica* (A.Br.) Fraser-Jenkins et Corley

[1977] which grows in the Caucasus and euxine region of Turkey and Iran. Among other hybrids of the group, Fraser-Jenkins also found there wild diploid *D. x initialis* = *D. caucasica x oreades* which by spontaneous chromosome doubling indeed gives rise to tetraploid *D. filix-mas* (Fraser-Jenkins et al. unpubl.) Table 4 shows that the phloroglucinol composition of *D. filix-mas* is in good agrrement with this origin. At

	origin	ploidy level	flavaspidic acid (5)	aspidinol (6)	para-aspidin (7)	desaspidin (8)	albaspidin (10)	phloraspin (11)	phloropyron (15)	filixic acid (19)	trispara-aspidin (20)	trisdesaspidin (21)	trisflavaspidic acid (23)	tetra-flavaspidic acid (26)	References
D.expansa	He	2x	+	+++	+++	-	(+)	-	+++	-	-	-	-	-	Widén et al.1970a *)
D.intermedia	Can	2x	+/++	+++	-	-	(+)	-	-	-	-	-	-	-	Britton & Widén 1974
	USA	2x	+	+++	-	-/+	(+)	-	-	-	-	-	-	-	Britton & Widén 1974
D.azorica	Az	2x	+	+++	-	-	(+)	-	-	-	-	-	-	-	Widén et al. 1975 a
D.maderensis	Md	2x	+	+++	-	-	+	-	-	-	-	-	-	-	Widén et al. 1975 a
Sum of D.expansa & maderensis	-	-	+	+++	++	-	(+)	-	++	-	-	-	-	-	
D.dilatata	He Br	4x	+	+++	+++	-	(+)	-	(+)	-	-	-	-	-	Widén et al. 1970a
D.campyloptera 1)	Can USA	4x	+/++	(+) /++	+++	-/(+)	+	-	+++	-	+	-	-	-	Widén & Britton 1971*) Britton & Widén 1974
D.oreades	I	2x	+++	-	-	-	-	-	-	++	-	-	+	+	Widén et al. 1971c *)
D.caucasica	Tu	2x	+	-	+++	++	-	-	-	+++	-	-	-	-	Widén et al. 1973 a *)
Sum of both	-	-	++	-	++	+	-	-	-	+++	-	-	(+)	(+)	
D.filix-mas	He	4x	+++	-	+	+	-	-	-	+++	-	-	(+)	(+)	Widén et al. 1971c and new results
D.villarii	I	2x	++	-	+++	+	+	-	-	+++	-	-	-	-	Widén et al. 1971 and new results
D.pallida	I	2x	+	-	+++	+	++	-	-	-	++	+	(+)	(+)	
Sum of both	-	-	++	-	+++	+	++	-	-	++	+	(+)	?(+)	?(+)	*)
D.submontana	Br	4x	++	-	+++	+	++	-	-	++	-	-	?(+)	?(+)	Widén et al. 1971 and new results
Sum of D.oreades & pallida	-	-	++	-	++	(+)	+	-	-	+	+	?(+)	?+	?+	
D.tyrrhena 2)	Co	4x	+++	-	+	-	++	(+)	-	++	+	-	(+)		Widén et al. 1971c

1) One sample of *D. campyloptera* from Quebec was devoid of phloroglucinols, further results see Widén et al. 1975 b.
2) Treated sub "*D. filix-mas* var. *rigidiformis*" in Widén et al. 1971: 2838.
*) and other abbreviations see table 2.

Table 4 — Examples of other allotetraploid *Dryopteris* species and their diploid ancestors in which chemistry reflects their natural relation. Main phloroglucinols (homologues not given) in the *D. dilatata, D. filix-mas* and *D. villarii* groups as well as in *D. thyrrena* as example of a combination between a member of the *D. filix-mas* and one of the *D. villarii* group.

the same time it may be useful to emphasize that the chemistry of secondary plant products alone can never really prove that an assumed evolutionary relation is correct. As pointed out earlier [Widén et al. 1973] the phloroglucinol spectra in *D. caucasica* and *D. villarii* ssp. *villarii* are virtually the same. So chemical analysis cannot distinguish which of these two otherwise quite distinct species is the appropriate candidate for the second ancestor of *D. filix-mas.*

D. submontana Fraser-Jenkins (= *D. villarii* ssp. *submontana* Fraser-Jenkins & Jermy). This tetraploid taxon was first reported from Great Britain by I. Manton [1950:65]. Cytological examination of experimental and natural hybrids [Panigrahi 1965, Roy 1967, Fraser-Jenkins & Gibby in prep.] strongly indicates it to be an allotetraploid once formed by chromosome doubling from a diploid hybrid of *D. pallida x villarii* (= *D. x vidae* Fraser-Jenkins & Gibby in prep.), although its allotetraploid nature has not yet been proved, however. The chemical results (table 4) are quite compatible with such an origin.

D. tyrrhena Fraser-Jenkins & Reichts. As a last example we give in table 4 results for *D. tyrrhena*, a species which can easily be (and often has been) confused with members of the *D. filix-mas* and *D. villarii* group. It has been shown that *D. tyrrhena* probably once arose by chromosome doubling in a diploid hybrid of *D. oreades* × *pallida. D. pallida* differs in phloroglucinol composition from *D. caucasica* only in the absence of filixic acid (*19*) and presence of albaspidin (*10*), trispara-aspidin (*20*) and traces of trisdes-aspidin (*21*). It is therefore understandable that the composition of phloroglucinol in *D. tyrrhena* is rather similar to that in *D. filix-mas*, but the small differences, presence of albaspidin (*19*) and trispara-aspidin (*20*), are exactly in the expected direction.

3.5. **Chemical analysis as a tool to differentiate species.**

To illustrate the useful contribution chemistry can make to taxonomic problems, particularly if combined with cytology, we give results for East-African *Dryopteris*, as a first example, see table 5. The work represents a joint effort to establish the true number of *Dryopteris* species growing in Kenya, based on material mainly collected by Faden et al. [see Widén et al. 1973b].

There is no agreement among the experts whether 5, 6, or 7 species grow there. *D. squamiseta* is not included in these figures (nor in table 5), because it was some time ago been transferred to *Nothoperanema* by Ching [1966]. While all workers agree that the first four species in table 5 are distinct and give rise to no particular taxonomic problems, there is no agreement about the other taxa, which can be summarized as *D. inaequalis* group (or complex). Some authors, particulary Schelpe [1970] and Kornas' [1979], make *D. pentheri* (Krasser) C.Chr. a synonym of *D. inaequalis* (Schlecht.) O.Ktze. Pichi Sermolli [in lett. 30.6.1971] and Faden [1974] accept them as distinct species. According to morphological, chemical and cytological results, the situation is even more complicated. There are at least four distinct taxa (accepted here as species) in this group. Besides of a tetraploid, tentatively called here *D. inaequalis*, and two diploids, called here *D. pentheri* and *D. schimperana* (A. Br.) C. Chr., there is an unnamed further tetraploid R.B. Faden No.

71-862. It is intermediate in morphology between *D. pentheri* and *D. schimperana* and the chemistry would fit very well the assumption of its being an allotetraploid (= amphidiploid) of these two. It must be emphasized that the names attached to the mentioned material (vauchers deposited in H) are provisional. According to Pichi Sermolli true *D. inaegualis* is restricted to S. Africa. If this is true, the taxon called so in this study must be renamed (see Jacobson 1978).

Among the taxa of table 5 *D. kilemensis* represent one of the few *Dryopteris* species being devoid of phloroglucinols. (There is, of course, the possibility that this is only true for the particular sample which could be analysed). As mentioned before, some individual samples of *D. carthusiana* and *D. expansa* were also devoid of phloroglucinols. *D. pentheri* is remarkable from the chemical point of view, it contains pentherin-I, a new compound which is optically active (!) and in this respect unique among phloroglucinols from ferns. It has so far been encountered otherwise only in the tetraploid "Faden No. 71-862". *D. inaequalis* showed the most complicated pattern of phloroglucinols with 10 components.

	ploidy level	flavaspidic acid (5)	aspidin (6)	para-aspidin (7)	desaspidin (8)	albaspidin (10)	phloraspin (11)	phloraspidinol (12)	margaspidin (13)	phloropyron (16)	methylene-bis-aspidinol (18)	filixic acid (19)	trispara-aspidin (20)	trisflavaspidic acid (23)	tetraflavaspidic acid (26)	pentherin-I
D. athamantica	2x	+	–	+++	–	(+)	–	–	–	–	–	–	–	+	–	–
D. callolepis	4x	+	+++	+++	(+)	(+)	–	–	–	(+)	–	–	–	–	–	–
D. manniana	4x	+++	–	–	+	+	–	–	–	–	–	++	–	+	+	–
D. kilemensis	2x	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D. inaequalis	4x	+	–	++	–	(+)	+	++	++	–	+	(+)	+	–	–	–
D. pentheri	2x	+++	–	–	–	–	–	–	–	–	–		–	+	–	++
D. schimperana	2x	+++	–	–	–	(+)	–	–	–	–	–	+	–	+	–	–
D. spec. Faden No. 71-885	4x	+++	–	–	–	(+)	–	–	–	–	–	+	–	+	–	++
Sum of D. pentheri and D. schimperana		+++	–	–		(+)	–	–	–	–	–	+	–	+	–	+

Table 5 — Showing semiquantitative composition (incl. new results) of the main phloroglucinols (homologues not mentioned) in eight *Dryopteris* species from Kenya. A hypothetical partial structure for pentherin-I [Widén et al. 1973: 2138] is based on reductive cleavage and its mass spectrum. It is the first phloroglucinol from fern found to be optically active.

3.6. **Dryopteris remota** (A.Br.) Druce. Finally we give an example of how chemistry helped in establishing the correct nature of *D. remota*, based on *Aspidium remotum* A. Braun [1850], a type collected in 1834 in the Northern Black Forest (deposited in B) and kept living in cultivation by Braun in several botanical gardens (Freibung i. Br., Karlsruhe and Leipzig) at least till 1873. The living material has since vanished. As shown by Manton [1938. 1950:71-75] the name was attached to two morphologically rather similar but distinct taxa: an apogamous triploid propagating normally from spores and a tetraploid sterile hybrid. As we know today, the former is widely distributed from Spain through Europe at least to Turkey and the Caucasus, missing in some areas and everywhere rare. The tetraploid hybrid was found only once [1854] by Huddard in England, introduced into cultivation by F. Clowes [1860] and, after some vegetative propagation, offshoots were distributed to some experts and botanical gardens, so that Manton was able to secure living material for her work from F. W. Stansfield. Unfortunately Stansfield's collections were lost after his death, but a big clump of the hybrid was found accidentally by C.R. Fraser-Jenkins still growing in the Botanical garden at Oxford and unequivocal material after checking of cytology was available for chemical analysis [Widén et al. 1976].

As mentioned above, the gross morphology of the triploid species and the tetraploid hybrid is very similar. In the living state the two taxa can easily be distinguished by counting their chromosomes and by checking the content of ripe sporangia. The apogamous triploid produced mainly good spores and the tetraploid hybrid only abortive material. Unfortunately chromosome counting is impossible in old herbarium specimens and checking of spores becomes increasingly unreliable with age and handling. In the triploid species the good spores are usually lost after such events and only the abortive material is left, or mainly so. This is the main reason why for a long time it was not established to which of the two taxa the name *D. remota* should be attached [see Reichstein 1965; Benl & Eschelmüller 1973]. After careful study of the type of *Aspidium remotum* it nevertheless became evident [Fraser-Jenkins et Reichstein, publ. in preparation] that it must be the triploid species. This also still grows quite near to the locus classicus in the Northern Black Forest [H. & K. Rasbach & T. Reichstein, publ. in preparation]. The tetraploid hybrid has therefore been given a new name *D.* ×*x. brathaica* Fraser-Jenkins et Reichstein [1978].

Fortunately, the two taxa contain rather different phloroglucinols (see table 6), and a small piece of rhizome of A. Braun's original material of his *Aspidium remotum*, collected more than 140 years ago was still available. In spite of the age of the sample, the chemical analysis gave a clear result, corresponding well with the triploid species (*D. remota*) in complete agreement with the morphological comparison.

	origin	ploidy level	flavaspidic acid (5)	aspidin (6)	para-aspidin (7)	desaspidin (8)	albaspidin (10)	phloraspidinol (12)	phloropyron (15)	filixic acid (19)	trisdesaspidin (20)	trisdesaspidin (21)	trisflavaspidic acid (23)	tetraflavaspidic acid (26)
D. carthusiana	SF	4x	+/++	+++	-/+++	++	++	-	-/+	-	-	+	-/+	-
D. filix-mas	He	4x	+++	-	+	+	-	-	-	+++	-	-	(+)	(+)
Sum of above		-	++	+	++	+	+	-	(+)	+	-	(+)	(+)	-/(+)
D. x brathaica	Br	4x	+++	+	+	+++	++	-	-	++	-	+	+	-
D. remota	He	3x	+	+	+++	(+)	(+)	(+)	-	-	++	(+)	(+)	(+)
Aspidium remotum (type)	Ge	-	(+)	++	+++	(+)	+	(+)	-	-	++	+		

Table 6 — Semiquantitative composition of *D. x brathaica* with its putative parents (*D. carthusiana* and *D. filix-mas*), *D. remota* (checked triploid) and *Aspidium remotum* A. Br. (type).

4. **Appendix. Analytical methods.** All temperatures are given in °Celsius (centigrades). It is clear that reliable conclusion depend on reliable analytical methods. Most phloroglucinols are relatively stable to acids but sensitive to alkaline reaction, oxidation, heat, and contact to many adsorbents like aluminium oxide (AlO_3) and even silica gel (SiO_2). Some deteriorate quicker than others.

4.1. **Thin layer chromatography (TLC).** This method introduced for separating phloroglucinols by von Schantz & Nikula [1962] is still the most convenient procedure for quick analysis, it has been slightly modified by Widén (1967) and Widén et al. (1970). If possible the results should be confirmed by preparative isolation of the pure compounds. Unbuffered SiO_2 plates can give erratic results and Schantz & Nikula [1962] recommed SiO_2 impregnated with citric acid phosphate pH 6, which is still the best procedure. For analysing mixtures not well separated under such condition • plates buffered to pH 4-8 according to the gradient technique of Stahl [1964] have been widely used during the last years [Haapalainen & Widén 1970, Widén et al. 1973b:2131; 1975a:883]. Widén et al. [1976a:1729-30], on the other hand, recommend homogenously buffered plates at pH 4.0 and 6.0. In some cases plates buffered to pH 8.0 can give additional information, but often they produce detoriation (see below). For very polar compounds (as 5, 23 and 26) plates buffered to pH 3 or even 2 may be useful and are harmless. So far SiO_2G (containing gypsum) has been used for such work. In the following we describe the preparation of SiO_2-H plates and their advantage in analysing phloroglucinols.

Preparation of buffers [see Widén et al. 1976a:1740-41]
0.2 M phosphate solution (III) = 35.5 g $Na_2HPO_4.2H_2O$ "Merck" in water, ad 1000 ml
0.1 M citric acid solution (IV) = 21.01 g cryst. citric acid ($C_6H_8O_7.H_2O$) "Merck" in water ad 1000 ml
buffer pH 8 = 97.3 ml III + 2.7 ml IV
buffer pH 6 = 63.2 ml III + 36.8 ml IV
buffer pH 4 = 38.6 ml III + 61.4 ml IV

Preparation of buffered SiO_2-H plates. 30 g Silicagel (Art. 7739) Kieselgel HF (Typ 60) für die Dünnschichtchromatographie, Merck) is mixed well in a blend with 70 ml buffer solution and a 0.25 mm thick layer of this paste is applied to the glass plates. These are first dried at room temperature for 30

Formula No.	Compound and colour after spraying with "fast blue salt B" (0.1% in water)		RF values System I pH 4	RF values System I pH 6	RF values System II pH 4	RF values System II pH 6	RF values System III pH 4	RF values System III pH 6
2-B	aspidinol-B	(violet)	0.37	0.48	0.40	0.43	0.87	0.73
3-B	fraginol-B	(yellow)	0.52	0.25	0.49	0.47	0.89	0.73
4-AP	norflavaspidic acid-AP	(brown violet)	0.37	0.03	0.54	0.17	1	0.70
4-BB	norflavaspidic acid-BB	(brown violet)						
5-AB	flavaspidic acid-AB	(brown violet)	0.07	0.0	0.19	0.02	0.72	0.31
5-BB	flavaspidic acid-BB	(brown violet)	0.12	0.0	0.27	0.02	0.85	0.45
6-AB	aspidin-AB	(yellow)	0.83	0.55	0.72	0.73	1	1
6-BB	aspidin-BB	(yellow)	0.85	0.70	0.72	0.78	1	1
7-AB	para-aspidin-AB	(brown)	0.75	0.31	0.67	0.67	1	1
7-BB	para-aspidin-BB	(brown)	0.81	0.47	0.66	0.69	1	1
8-AB	desaspidin-AB	(orange red)	0.55	0.20	0.51	0.52	1	1
8-BB	desaspidin-BB	(orange red)	0.57	0.23	0.53	0.54	1	1
9-AB	orthodesaspidin-AB	(orange)	0.74	0.54	0.59	0.68	1	1
9-BB	orthodesaspidin-BB	(orange)	0.77	0.69	0.63	0.74	1	1
10-AA	albaspidin-AA	(red)	0.82	0.58	0.68	0.73	1	1
10-AB	albaspidin-AB	(red)	0.84	0.69	0.70	0.78	1	1
10-BB	albaspidin-BB	(red)	0.85	0.75	0.70	0.79	1	1
11-BB	phloraspin-BB	(brown)	0.32	0.26	0.45	0.43	0.81	0.71
12-BB	phloraspidinol-BB	(violet)	0.32	0.37	0.53	0.53	0.87	0.77
13-BB	margaspidin-BB	(brown) *)	0.55	0.45	0.52	0.50	0.89	0.80
14-BB	methylene-bis-desaspidinol-BB	(violet)	0.27	0.31	0.51	0.39	0.87	0.81
15-BB	phloropyron-BB	(red)	0.77	0.53	0.66	0.68	1	1
16-BB	phloraspyron-BB	(purple)						
17-BB	aemulin-BB	(light brown) *)	0.61	0.48	0.56	0.49	1	0.86
18-BB	methylene-bis-aspidinol-BB	(violet)	0.54	0.58	0.72	0.72	1	0.96
19-ABA	filixic acid-ABA	(brown)	0.64	0.11	0.70	0.53	1	1
19-ABB	filixic acid-ABB	(brown)	0.73	0.18	0.72	0.62	1	1
19-BBB	filixic acid-BBB	(brown)	0.81	0.30	0.75	0.73	1	1
20-BBB	trispara-aspidin-BBB	(brown)	0.29	0.01	0.82	0.55	1	1
21-BBB	trisdesaspidin-BBB	(light brown)	0.25	0.01	0.68	0.37	1	1
22-BBB	trisaspidin-BBB	(red)						
23-BBB	trisflavaspidic acid-BBB	(orange)	0.01	0.01	0.14	0.01	0.46	0.22
24-BAB	trisaemulin-BAB	(brown)	0.72	0.23	0.81	0.80	1	1
24-BBB	trisaemulin-BBB	(brown)	0.77	0.39	0.85	0.82	1	1
25-ABBA	methylene-bis-norflavaspidic acid-ABBA	(brown)	0.35	0.02	0.72	0.37	1	1
25-BBBB	methylene-bis-norflavaspidic acid-BBBB	(brown)						
26-BBBB	tetraflavaspidic acid-BBBB	(orange)	0.0	0.0	0.07	0.0	0.28	0.16

Table 7 — RF values in TLC on silica gel "HF (Typ 60) Merck". 1 = nearly to front.
*) At pH 6 an additional weak spot at RF 0.01 and 0.07 visible in system I and II respectively.

minutes and then activated at 1050 for 30 minutes. This material gives better spots, often better separation of spots and rather different RF values than SiO_2-G (containing gypsum) and, being fluorescent, allows localisation of spots under UV light if necessary, without subsequent spraying. In table 7 we give the RF values for most of the known *Dryopteris* phloroglucinols on such plates buffered for pH 4 and pH 6 in the following three systems.

Solvent systems. System I = mixture of methanol 10 g, isopropylether (freshly distilled b.p. 67-68° C), 35 ml and cyclohexane 55 ml. Isopropylether decomposes on standing to isopropanol and acetone and must therefore be freshly distilled before mixing. System II = mixture of chloroform-hexane-ethanol (45:45:10). System III = chloroform-hexane, ethanol-glacial acetic acid (45:35:16:4) freshly mixed. For very slow mooving compounds a System IV was useful with 6 % of glacial acetic acid.

Ascending chromatography. The RF values in table 7 were obtained after a single run. When the front had moved 15 cm the plates were dried in air at c. 20° for 5 minutes. Resolution of an otherwise too slowly mooving compound is better after addition of acetic acid than by chromatographing twice. Spots were visualized first in the dark chamber under UV light and afterwards by spraying with a freshly prepared 0.1 % solution of "fast blue salt" ("Echthlausalz B" Merck) = tetrazotised di-ortho-anisidine in water [A.Penttilä & J. Sundman, J. Pharm. & Pharmacol. 13:531 (1961)]. The pH 8 plates should be used with caution since many phloroglucinols are deteriorated under such conditions. An absorbent can be accepted as harmless if a drop of solution of a sensitive compound (e.g. 13 or 23) is chromatographed soon after application and another sample of the same compound is applied 1-2 hours before and left in air on the same plate, provided both have the same running properties [Widén et al. 1976:1740 footnote].

B and P homologues (like *7-PB* and *7-BB* etc.) in general move together in TLC. From these the A homologues can usually be separated (see *5-AB, 5-BB, 6-AB, 6-BB, 7-AB, 7-BB* etc. in table 7, also Widén 1970, Acta bot. fenn. *91*:1).

Remark. In the same way a slightly different type of Silicagel (Art. 7741, Kieselgel HF 254+366 (Typ 66) für Dünnschichtchromatographie Merck) can be used. It has the advantage that detection under UV light of longer wave length which is less agressive can be used. The RF values are slightly different.

The given absolute RF values are only intended to be approximate figures. They may vary appreciably in each experiment and have to be corrected against pure reference compounds. For identification of spots in mixtures the parallel running of reference compounds on the same plate is indispensable.

As evident from the given RF values not all compounds can be differentiated with confidence in these system. Sometimes two compounds with similar RF values (e.g. *6*, *10* and *19*) can be differentiated by the colour produced after spraying but this becomes unreliable in mixtures or if both give similar colours (e.g. *7* and *24*). For such cases it is advisable to use other systems or different methods. It is possible first to make a crude separation on buffered SiO_2 plates (at pH 4 or 6), and then visualized the spot under UV light (i.e. without spraying). Scratch out the material so enriched, moisten it slightly with dilute (2N) aqueous HCl, and elute with pure ether or a little chloroform. The residue of the compound solution is rechromatographed on polyamide or precoated SiO_2 in reversed phase system or in HPLC.

For *TLC on polyamide* we used commercially available plates: Art. 5555 DC-Alufolien Polyamid 11F 254 20x20 cm Merck. As solvent: methanol-methylethylketone-benzene-glacial acetic acid 24:30:44:2. Detection under UV light (254 nm max), spraying with fast blue salt B give no colour on this hydroophobic material (polymer of amino undecanoic acid). Table 8 gives a few RF values to show some pairs (e.g. albaspidin and filixic acid, etc.) which do not separate well on SiO_2 but fairly well on polyamide -11. Unfortunately sometimes failing was observed on such plates.

For reversed phase we also used commercial plates: Art. 13725 HPTLC-Fertigplatten RP-sF 254 S für die Nano-DC 10x10 cm Merck. Solvent was methanol-water 95:5. Detection under UV light as well as by spraying. Some results see table 8. Homologues are usually not separated in this system.

4.2. **Other methods.** All methods that involve high temperatures, such as gas chromatography (GLC) (see Pyysalo & Widén 1979a, b), cannot be of more than limited use due to detoriation.

High performance liquid chromatography (HPLC). This may become the method of choise for analysing mixtures of phloroglucinols when further worked out. Widén et al. [1980].

Mass spectrometry. In spite of the fact that all presently available methods for mass spectrometry involve considerable heating of the sample, most valuable results both for identification and structure determination can be obtained even with conventional electron impact (EI) mass spectra [Lounasmaa et al. 1971a; 1972; 1973; Lounasmaa 1973]. The method is also very efficient for analysing mixtures of homologues and degradation products. With gentler methods using field ionisation (FI) (unpublished), chemical ionisation (CI) [Lounasmaa & Varenne 1978; Lounasmaa 1979] and field desorption (FD)

(unpublished), excessive fragmentation can be avoided. FD seems to be particulary suitable for difficult compounds, and distinct molecular peaks could often be observed which were not visible or faint with other methods.

4.3 **Reductive cleavage under mild conditions.** As mentioned, this reaction is used for structure determinations but also for analysing mixtures of homologues, etc. Details given in Widén et al. 1970:2186:5 mg of material and 10 mg Zn dust are warmed with stirring in 20 ml of 5 % aqueous NaOH for 5 minutes at 100°. After cooling, the insoluble particles are eliminated by filtration, the clear solution is acidified with HCl and extracted with ether. The ether solution is dried over Na_2SO_4 and evaporated. The residue is analysed in buffered systems [for RF values and analytical data see Widén et al. 1973:2132-33, 2941], acyl-filicinic acids (*36* and homologues) in PC at pH 4.0 and aspidinols (2 and homologues) in PC at pH 8.6 with solvent cyclohexane-chloroform 1:1 (v/v) [Penttilä & Sundman 1961, 1963a] giving good separation, and for acyl-phloroglucinols (27 and homologues) ph 6, solvent n-hexane-chloroform-ethanol-45:45:10 [Widén et al. 1973]. The two latter groups are not separated in PC and only partially in TLC and are best further examined in mass spectra or in glass capillary GLC as diethylethers (Pyysalo & Widén 1978a).

4.4 **Reductive cleavage under vigorous conditions.** This method is sometimes more convenient for structure determinations and analysing mixtures of homologues. Details given in Widén et al 1973: 2141-42 and Lounasmaa et al. 1974. 50 mg material and 100 mg Zn dust are boiled underr eflux with 20 ml 15 % aqueous NaOH for 24 hours. After filtration the clear solution is acidified with 25 % HCl and extracted 3 times with 20 ml of ether. The ether solution is dried over Na_2SO_4, carefully reduced to a small volmume (to avoid losses of volatile acids) and partly used for ingestion into an appropriate gas chromatograph adapted for analysing homologous fatty acids. Acetic, propionic, n-butyric, isobutyric and n-valeric acid are used as standards. The ether solution can also be used to analyse the deacylated homologues of *2*, *27*, *31* and *36* (Lounasmaa et al. 1974b). The method can be used for a rapid and reliable analysis of pure crystalline phloroglucinols for building units and crude "filicins" for homologues.

4.5. **The standard method for preparative isolation.** The method used so far for isolation or at least concentration of phloroglucinols for subseguent identification by TLC includes the following steps.

Rhizomes (if possible washed free of soil) or stipe bases (as second choice) are dried in air at c. 20° for several weeks or at c. 40° for 4-8 days and powdered.

The powder is exhaustively extracted with pure (peroxide free) ether. Evaporation of the ether solution leaves a residue = "crude ether extract", a viscous, honey like mass containing all phloroglucinols, plus larger quantities of fatty acids, neutral fats, waxes, steroids, etc. (see Huhtikangas et al. 1980). Provided pure, peroxide free ether is used, no deteriation occurs. The mixture can be analysed directly by TLC or HPLC.

To concentrate the phenolics further (up to c. ten-fold), the crude ether extract has usually been treated with MgO and water to transform the fatty acids into insoluble Mg-salts and the phenolics into water soluble Mg-salts. Filtration and washing with water leave an insoluble MgO cake containing in addition to excess MgO and other possible additives (Na_2SO_3 see below), the insoluble Mg-salts and the bulk of neutral material. The phenolics go into the aqueous solution and are precipitated with acid (HC1). The material so obtained is often called "MgO-filicins". This step usually gives losses by incomplete reaction (mechanical occlusion) and degradation. The latter can be avoided in part by quick performance, low temperature, and addition of Na_2SO_3 as "stabilizer" [Ackermann & Mühlemann 1946]. Usually it is possible to get additional amounts of phenolics by treating the MgO cake with Ba $(OH)_2$ [Widén & Britton 1971; Widén et al. 1976: 1740] but this may involve further detoriation. The precipitates obtained from the aqueous solution of this step are often called crude "$Ba(OH)_2$-filicins". The "MgO" - and

"Ba $(OH)_2$" -filicins represent mixtures of essentially pure phenolics, which can be analysed further by TLC or HPLC, and often give cleaner results than the crude ether extracts.

Details of procedure. 1 g of crude ether extract is well mixed by grinding in a mortar with 2 g of MgO and 0.5 g crystalline Na_2SO_3, further ground with 7 ml 1 % Na_2SO_3 in water at 0°, and quickly filtered with suction into 10 ml cooled 25 % aqueous HCl. The "filicins" precipitate as a light yellow amorphous powder. The insoluble residue is again well ground with 7 ml 1 % Na_2SO_3 at 0° and filtered into the acid suspension. THis is repeated two more times.

The yellowish precipitate is either filtered with suction, washed with water and dried in vacuo to yeld the crude "MgO-filicins", or extracted with ether and the ether solutions dried over Na_2SO_4 and evaporated.

The last MgO filter cake can be treated in exactly the same way by grinding with 2 g Ba $(OH)_2.8H_2O$ + 0.5 g crystalline Na_2SO_3 and further proceeding as above to give an additional amount of phenolics as "Ba $(OH)_2$-filicins". This usually contains most of the less acidic phloroglucinols like phloraspin (*11*), phloraspidinol (*12*), margaspidin (*13*), methylen-bis-desaspidinol (*14*) and methylen-bis-aspidinol (*15*) if present.

Column chromatography. For the isolation of crystalline compounds or achievement of partial separation to make TLC more reliable, the crude "filicins" have usually been chromatographed on columns with an about 30 fold amount of unbuffered SiO_2 (Silicagel) für Säulenchromatographie "Merck", Korngrösse 0.05 - 0.2 mm). Details are given for example in Widén et al. 1976 and are not repeated here. Appreciable degradation or rearrangements may occur in this step, particularly if repeated chromatography is necessary to achieve sufficient separation. The undesired reactions can be minimized by quick work and selecting an appropriate quality of absorbent, but best by pretreating the sample with buffers or other protecting material and subsequently reactivating by heating in vacuo (see below sub 4.6.).

4.6. **Attemps to improve methods.** We give here preliminary results for a new method that avoids the two critical steps, i.e. MgO or Ba $(OH)_2$ treatment and chromatography on columns of unbuffered SiO_2. Application to *D. filix-mas* is given as an example. This plant is known to contain filixic acid (*19*) and flavaspidic acid (*5*) as the main phloroglucinols together with minor amounts of other components [Widén et al. 1971]. The method proceeds as follows.

Extraction of powdered rhizome with pure ether is the same as in the standard method. The ether solution, after partial concentration is freed from cations by shaking with aqueous HCl. The dried ether solution is evaporated and this crude, cation free ether extract is counter current distributed between hexane (5 layers) and 95 % methanol + 5 % water mixture (c. 30 or 60 layers). The less polar compounds, i.e. all the fatt material together with all filixic acid (*19*) and some minor constituents stay in the hexane phases while the more polar material, i.e. the bulk of flavaspidic acid with other more polar compounds, go into the methanol phase.

The material from the hexane phases is then chromatographed on purified hydrophylic polyamide (Polycaprolactame). We used a commercially available product "SC 6" (see below). It gives a fairly good separation of fatty material mixed with a small amount of filixic acid. The bulk of the latter can be recovered by direct crystallisation.

The material from the methanol phases cannot be chromatographed on polyamide "SC 6" because it is too firmly adsorbed and can only be recovered from the column by addition of acetic acid or other drastic methods. Chromatography on microcrystalline cellulose (Avicel "Merck") gave fairly good results and columns prepared from SiO_2 buffered for pH 6 and reactivated at 105° after drying gave good separation with no detectable detoriation. Unfortunately some citric acid is eluted

particularly with the more polar fractions; its elimination necessitates an additional operation.

This method not only gave appreciably higher yields of crystalline filix-acid and flavaspidic acid from *D. filix-mas* than the standard procedure, but also immediately showed that trisflavaspidic acid (*33*) and a new compound which we tentatively formulate as tetraflavaspidic acid (*26*), so far not detected in *D. filix-mas*, are present in this species.

Details of the procedure. General precautions. Commercial pure solvents were distilled before use. Ether in particular must have been freshly distilled not longer than 2 days before and kept in the dark. (For liquid extraction and counter current distribution selected separatory funnels were used in which the ground glass stoppers keep sufficiently tight to allow clean separation during the work with only a drop of water or methanol as lubricant, i.e. without any fat, silicone or other grease. During storage in the dry state the stoppers of the funnels must he protected with a strip of paper to assure their later removal.

Purification of hydrophylic Polyamide for column chromatography. Commercial "MN-Polyamid SC 6 (Polycaprolactam) für die Säulenchromatographie Machery Nagel & Co" was well washed at room temp. with ethylacetate, methanol (twice), acetone (3 times) and ether and dried in vacuo at 20°.

Purification of microcrystalline cellulose. Commercial: "Art. 2331 Cellulose microcristallin "Avicel" für die Säulenchromatographie Merck" was washed at room temp. with water (twice), methanol (3 times) and acetone, dried in vacuo (0.1 Torr and 70-80°) for one hour.

Preparation of buffered silica gel for column chromatography. Commercial silica gel: "Art. 7734 Kieselgel Korngrösse 0,063 - 0,200 mm (70 - 230 mesh AST M) für die Säulenchromatographie Merck" was washed with water, methanol, chloroform, acetone and ether, dried in vacuo, finally at 100°. 30 g of this material was well mixed with 60 ml buffer solution pH 6 (see above) and dried in vacuo finally at 60-70° for one hour. -The fractions obtained from columns prepared with this material after evaporating in vacuo have to he dissolved in ether (or ether/chloroform mixture) and washed with water to eliminate citric acid. The washed ether layer gives the citric acid free material. This is a complication so far unavoidable.

Preparation of rhizomes. A large plant was dug out (TR4167, cult. in garden of J.v.Euw since many years) and the fronds and fibrous roots were cut off. The rhizome was well washed hy brushing under running water to eliminate soil (weight of clean rhizome 2.7 kg) and dried on a sieve in a slow stream of warm air at 40-50° for several days. When reaching a constant weight (660 g = 24,2 %) the dry rhizomes were broken and powdered in a mill.

Extraction. 260 g dried powdered rhizome was warmed 1200 ml ether under reflux for 10 minutes and after cooling filtered under slight pressure. The filter cake was further extracted 10 times in the same manner. The last extract was virtually free from phenolics. The combined solutions were concentrated to c. 200 ml and this concentrate washed 3 times with 10 ml N aqueous HCL and 3 times with water. The aqueous phases went through 3 more funnels with 100 ml ether each to reetract traces of phenolic material. -As a check the final acid aqueous phases were combined, evaporated in vacuo and the residue dried over KOH at 0.1 Torr, leaving 296 mg "salts" (dark brown mass, soluble in methanol but poorly soluble in water and in dilute HCl) which were not further investigated. The ether layers were dried over Na_2SO_4 and evaporated giving 23.93 g (= 9.2 % of dry rhizome) "crude cation free extract".

Counter current distribution. This was performed by manual shaking in 5 separatory funnels. 12.47 g "crude cation free extract" (= 1.35 kg of dry rhizome) was transferred into the first funnel with 15 ml 95 % methanol 5 % water mixture and 200 ml hexane and shaken. After separation the methanolic layer was transferred to the second funnel where it was shaken with 100 ml fresh hexane, then to the third, fourth and fifth. Thereafter 15 ml fresh 95 % methanol was again introduced into the first funnel and after shaking and separation transferred to the second, and so on. The whole procedure was repeated until 30 methanolic phases have passed.

The five remaining hexane layers (after 30 methanolic washings) were dried over Na_2SO_4 and evaporated giving 8.69 g "hexane soluble" material (= less polar) containing fatty acids, etc., all the filixic acid, and still a small amount of flavaspidic acid, etc.

After chromatography on cellulose or polyamide -6, 513 mg crystalline filixic acid (0.035 % of rhizome) was obtained. This amount is appreciably more than in the standard method. Filixic acid (and other phenolics) can be obtained from the material (8.7 g) by dissolving it in 100 ml ether, shaking twice with 5 ml 20 % cold $KHCO_3$ solution and twice with water (5 ml), and repeating the operation 3 times. Separation may be slow but the phenolics (c. 2 g) are recovered from the aqueous layers after acidification and extraction with ether. They contained all filixic acid which partly crystallised directly and the rest after chromatography. The yield was similar; no deterioration was observed.

Flavaspidic acid present in this extract can be recovered, with considerable impurities, from polyamide -6 columns by addition of some 10 % acetic acid as solvent, and obtained in crystal from after chromatography on cellulose.

The combined methanolic layers were freed from methanol in vacuo and the remaining aqueous suspension extracted 3 times with ether. The ether solution dried over Na_2SO_4 and evaporated gave 3.55 g "methanol soluble" material (more polar) containing the bulk of flavaspidic acid, and tris- and tetraflavaspidic acid. 330 mg crystalline flavaspidic acid (= 0.0244 % of dry rhizone) as well as crystalline mixtures (c. 30 mg) of tris- and tetraflavaspidic acid were obtained after chromatography on microcrystalline cellulose. The latter two compounds were separated on SiO_2, buffered at pH 6 with the addition of a small amount of acetic acid to the eluting solvent.

To get additional information on the distribution behaviour we further extracted in one experiment the "hexane soluble" material (8.7 g), remaining after the 30 methanol extractions, 30 times with 95 % methanol in the same manner as before. The operation was repeated 3 times (150 extractions altogether).

Extractions 31-60 gave 980 mg "methanol soluble" material still containing flavaspidic acid, etc. From this 15 mg crystalline flavaspidic acid, m.p. 153-155°, could be obtained after chromatography on cellulose.

Extractions 61-90 gave 320 mg "methanol soluble" material still showing some flavaspidic acid in TLC, but no pure material could be isolated. Extractions 91-120 (344 mg) and 121-150 (289 mg) were free of flavaspidic acid and contained only small amounts of other phenolics, obviously other extractives. After these 150 methanol washings 7.60 g "hexane soluble" material was regenerated, still containing all the original filixic acid.

From this one can conclude that 30 extractions give a very useful separation, 60 may improve the result, but further are usually not necessary.

Riassunto
I più importanti fluoroglucidi ("filicine") isolati finora dai rizomi di *Dryopteris* vengono discussi. Lo studio chimico dei rizomi di *Dryopteris* (assieme ai metodi classici e citologici) può risultare utile alla soluzione di problemi tassonomici, particolarmente nei casi seguenti: 1. Identificazione di esemplari d'erbario altrimenti carenti. 2. Classificazione di taxa critici nell'ambito di gruppi difficili. 3. Comprensione delle relazioni naturali, ed in particolare identificazione dei parenti in gruppi ibridi e dei tipi ancestrali in specie allopoliploidi. I metodi analitici vengono valutati criticamente.

Zusammenfassung
Die wichtigsten, bisher aus den Rhizomen von *Dryopteris*-Arten isolierten Phloroglucide ("Filicine"), werden zusammengestellt. Die chemische Untersuchung von *Dryopteris*-Rhizomen kann (zusammen mit klassischen sowie cytologischen Methoden) bei taxonomischen Problemen besonders in folgenden Fällen von Nutzen sein. 1. Identifizierung sonst ungenügender alter Belege. 2. Abgrenzung kritischer Taxa in einem schwer unterscheidbaren Formenkreis. 3. Als zusätzliche Kriterien zur Feststellung der Verwandtschaft, besonders auch über mögliche Eltern von Hybriden und Vorfahren allopolyploider Arten. Die analytischen Methoden werden kritisch gewertet.

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