Phytochemistry, 1970, Vol. 9, pp. 575 to 579. Pergamon Press. Printed in England.

BIFLAVONES OF SOME *CUPRESSACEAE* PLANTS*

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(*Received* 28 *June* 1969, *in revised form* 4 *August* 1969)

Abstract—Two solvent systems, viz. toluene:pyridine:acetic acid (10:1:1) and toluene:DMF:acetic acid (10:1:1) have been found to be useful for the TLC study of biflavones in conifer extracts; these solvents have also been used for obtaining chromatographically pure biflavones and their methyl ethers by preparative TLC and "dry column" chromatography. Eight plants of the *Cupressaceae* family have been investigated for biflavones. Amentoflavone, cupressuflavone and hinokiflavone are found to co-occur in four *Cupressus* species although *c. sempervirens* contain only amentoflavone and cupressuflavone. *Biota orientalis* is found to contain hinokiflavone, quercetin and myricetin. Cupressuflavone and amentoflavone are invariably components of the genus *Cupressus* and may serve as taxonomic markers.

THE ESTABLISHMENT[1] of the structure of ginkgetin (I), discovered in the leaves of *Ginkgo biloba*, led to an extensive search for biflavonoids in plant sources.[2–4] Three basic types have been recognized, viz. amentoflavone (II), cupressuflavone (III) and hinokiflavone[5] (V); a large number of their partial methyl ethers have also been identified.[6–10] Recent interesting discoveries are the occurrence of optically active forms of cupressuflavone tetramethyl ether[11] and amentoflavone tetramethyl ether[12] in *Araucaria cookii*; the identification of two methyl ethers of agathisflavone (6,8″-biapigeninyl) in *Agathis palmerstonii* has also been reported.[13]

During our investigations of the Indian conifers it was frequently noticed that the biflavones which were "purified" by crystallization or through derivatives were not single entities; careful TLC examination of the compounds and their methyl ethers revealed the presence of other substances, some of which did not correspond to any of the known flavonoids or biflavonoids. Similar behaviour was also shown by samples of natural biflavones and their methyl and ethyl ethers supplied by other workers. The present study was undertaken to devise suitable TLC techniques for a rapid survey of the flavonoid and biflavonoid

*Part IV in the series "Biflavonoids".

[1] K. NAKAZAWA, *Chem. Pharm. Bull.* **11**, 223 (1963).
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[5] K. NAKAZAWA, *Chem. Pharm. Bull.* **16**, 2503 (1968).
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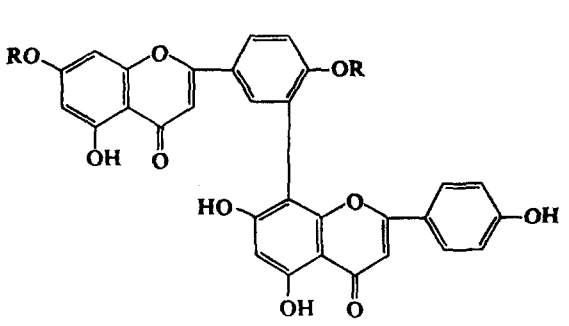
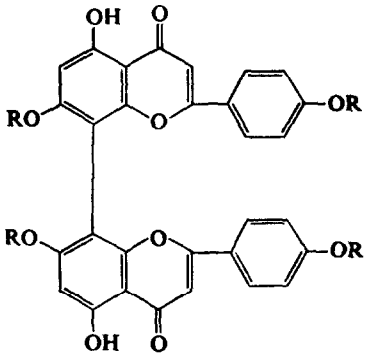
[10] H. MIURA, T. KIHARA and N. KAWANO, *Tetrahedron Letters* 2339 (1968).
[11] M. ILYAS, J. N. USMANI, S. P. BHATNAGAR, M. ILYAS, W. RAHMAN and A. PELTER, *Tetrahedron Letters* 5515 (1968).
[12] A. PELTER, R. WARREN, M. ILYAS, J. N. USMANI, S. P. BHATNAGAR, R. H. RIZVI, M. ILYAS and W. RAHMAN, *Experientia* **25**, 350 (1969).
[13] A. PELTER, R. WARREN, J. N. USMANI, R. H. RIZVI, M. ILYAS and W. RAHMAN, *Experientia* **25**, 351 (1969).

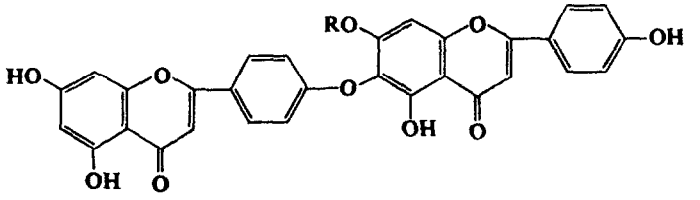
pigments in the extracts of conifer leaves and to isolate the minor biflavonoids present. The results of our studies on a number of Indian *Cupressaceae* are described in the present communication.



(I) R = CH_3
(II) R = H



(III) R = H
(IV) R = CH_3



(V) R = H
(VI) R = CH_3

It was earlier reported[2] that the leaves of *Cupressus funebris*, *C. arizonica* and *Biota orientalis* contain only hinokiflavone; more recently Miura and Kawano[9] found that *C. arizonica* and *C. goveniana* also contain cupressuflavone. We have now examined the leaves of the following species: *C. torulosa* Don., *C. sempervirens* Linn., *C. sempervirens* var. *stricta* Aiton, *C. funebris* Endl., *C. goveniana* gord., *C. glauca* Lam K. (syn. *C. lusitanica* Miller), *Biota orientalis* Endl., *B. orientalis* var. *Mexican* Dümmer. The last was from Kabul, Afghanistan. The leaves were extracted with ethanol and the pigments collected from the alcoholic concentrates in fractions as they were deposited. These were used for TLC examination and for the isolation of pure compounds.

TLC Screening of the Pigments

Preliminary TLC examination was carried out on silica gel in the following solvent systems:

(1) toluene:pyridine:AcOH (10:1:1) and (20:1:1);
(2) toluene:DMF:AcOH (10:1:1);
(3) toluene:EtOAc:AcOH (20:4:1);
(4) toluene:HCOOEt:HCOOH (5:4:1).[14]

[14] N. KAWANO, H. MIURA and H. KIKUCHI, *J. Pharm. Soc. Japan* **84**, 469 (1964).

The best resolution was obtained with solvent systems 1 and 2 and by developing the TLC plate repeatedly with the same solvent (multiple development). The hydroxy compounds and partial methyl ethers were located with ethanolic ferric chloride spray (2%), and fully methylated (and ethylated) compounds by their characteristic (blue, blue-green, yellow-green) fluorescence in u.v. light; the 5-hydroxy compounds appeared as dull-red spots and the fully hydroxylated compounds as dark-brown spots. The acetates were made visible as yellow spots by spraying with 25% sulphuric acid.

The crude mixtures were examined using known biflavonoids (and flavonoids) as standards for comparison. Agreement in R_f values at this stage may be coincidental; for example it was observed that cupressuflavone and amentoflavone sometimes did not separate well and appeared as a single spot. Therefore the pigments were methylated and again screened. A quick and convenient method of methylation which can be used on a small scale is described in the Experimental section. Authentic samples of biflavone methyl ethers (complete and also partial methyl ethers having the 5-hydroxyl groups free) were used as markers. As a further check the acetates were prepared and also examined by TLC. Such detailed cross-checking was found to be necessary in order to be certain about the identity of the various components and also to locate new substances.

In order to distinguish between monomeric (simple) flavones and flavonols the following procedure was employed: the TLC plate was first sprayed with a saturated solution of neutral lead acetate in 95% ethanol followed by 2 N ammonia; flavones appear as yellow spots whereas the flavonols give distinct pink or red spots. It is known that the dihydroflavonols and flavonol-3-*O*-methyl ethers give characteristic pink colours with zinc and hydrochloric acid. This colour reaction has now been adapted for TLC purposes (see Ref. 15). After the TLC plate has been developed it is sprayed with a suspension of zinc dust in acetone, followed by acetone–hydrochloric acid (1:1). The flavones and flavonols appear as yellow spots and the dihydroflavonols as pink spots; when methyl ethers are used pink spots appear with dihydroflavonol methyl ethers and flavonol methyl ethers. Quercetin and myricetin could be thus detected in *B. orientalis* extracts and was confirmed by isolation of the compounds and preparation of derivatives.

Isolation of Chromatographically Pure Biflavones

The usual methods for the isolation of pure biflavones is by crystallization of the compounds themselves or their derivatives, generally acetates. Pure ginkgetin was obtained[6] by taking advantage of the sparing solubility of its potassium salt. Counter-current distribution has been also used in some cases.[10] However, these methods do not always give chromatographically homogeneous substances. Further, these techniques are not convenient for the isolation of biflavones which are present only in small quantities. In the present study it has been found that multiple development TLC technique is capable of resolving compounds having close R_f values. We have now used this method to isolate pure biflavone methyl ethers by preparative TLC. Some new compounds which do not seem to correspond to the methyl ethers of any of the known biflavones have also been isolated in small amounts. These are being studied further.

Preliminary experiments have also been carried out on the use of "dry column" chromatography[16] for the isolation of pure biflavones in larger amounts. This method has been claimed to have the resolving power of TLC. Using solvent system 2 it was possible to

[15] G. M. BARTON, *J. Chromat.* **34**, 562 (1968).
[16] B. LOEV and M. M. GOODMAN, *Chem. Ind.* **48**, 2026 (1967).

isolate amentoflavone, cupressuflavone, hinokiflavone and isocryptomerin (VI) from the extracts of *C. funebris*. This technique is being examined further.

The main findings of the present study are shown in Table 1.

TABLE 1. DISTRIBUTION OF BIFLAVONES IN *Cupressaceae*

Species	Compound present*						
	A	C	H	IC	Api	Q	M
Cupressus sempervirens	+	+	−	−			
C. sempervirens var. *stricta*	+	+	−	−			
C. glauca	+	+	+	−			
C. goveniana	+	+	+	−			
C. funebris	+	+	+	+			
C. torulosa	+	+	+	−	+		
Biota orientalis	−	−	+	−	−	+	+
B. orientalis var. *Mexican*	−	−	+	−	−	+	+

* A = amentoflavone; C = cupressuflavone; H = hinokiflavone; IC = isocryptomerin; Api = apigenin; Q = quercetin; M = myricetin.

The present investigation has revealed that the *Cupressus* genus is rich in biflavones, particularly cupressuflavone; Miura and Kawano[9] found *C. arizonica* also contains this substance. It appears to be the characteristic biflavone of this genus and along with amentoflavone may serve as a useful taxonomic marker. Kawano[17] has earlier concluded that hinokiflavone occurs in *Cupressaceae* without exception; however, in the present study this compound could not be detected in two samples of *C. sempervirens*. The co-occurrence of all the three basic members of the biflavone group in some of the *Cupressus* plants is of biogenetic significance; further, the presence of apigenin in *C. torulosa* is highly suggestive. The previous finding of Sawada that *B. orientalis* contains hinokiflavone is confirmed (see also Ref. 9); two samples now studied contain, in addition, quercetin and myricetin. The occurrence of the partial methyl ethers of hinokiflavone in this plant is indicated; but no amentoflavone or cupressuflavone could be detected.

EXPERIMENTAL

TLC and "dry column" chromatography were done on silica gel supplied by the National Chemical Laboratory, Poona. The solvent systems employed and the methods of locating the compounds have already been mentioned. All compounds were identified by m.p., m.m.p., i.r., co-TLC and preparation of derivatives.

General Method of Extraction

The air-dried leaves were exhaustively extracted with hot EtOH in a Soxhlet. The solvent was removed and the concentrate repeatedly treated with light petroleum to remove chlorophyll and waxes. After keeping at 5°, the concentrate deposited a yellow solid which on boiling with EtOH gave a crisp powder (crop 1). By further concentration, further crops were collected and examined separately. The final mother liquors were also examined by TLC using solvent systems 1 and 2 (see text) and multiple development.

In the case of *Biota orientalis*, no solid separated from the extracts and fractionation was through neutral and basic lead salts. Further separation and identification of the individual components was carried out as described below.

[17] N. KAWANO, *J. Pharm. Soc. Japan* **80**, 1647 (1960).

Rapid Methylation Procedure

The crude pigment (*ca.* 25 mg) was suspended in EtOH (2 ml) and Me_2SO_4 (2 ml) added. KOH (40%, 6 ml) was added dropwise with shaking to the ice-cooled mixture until alkaline. After 1 hr H_2O (20 ml) was added and the product extracted with $CHCl_3$. The extract was washed (H_2O), dried (Na_2SO_4) and the solvent was removed. The residue was used for TLC examination. It was found that a mixture of the full and partial (with the 5-hydroxyl groups free) methyl ethers was usually formed. This is an advantage since definite identification of a compound could be done on the same chromatogram employing authentic specimens of its complete and partial methyl ethers.

Preparation of the Complete Methyl Ethers

The pigment (0·5 g) was dissolved in dry dioxan (200 ml), Me_2SO_4 (10 ml) and K_2CO_3 (50 g) added and the mixture was refluxed till the $FeCl_3$ reaction became negative (about 120 hr). At intervals small amounts of both reagents were added. The crude methyl ether was isolated as usual and was used for preparative TLC in solvent 1. The individual bands were located in u.v. light, separately collected and extracted with boiling alcohol; after removal of the solvent the pure methyl ethers were crystallized from a suitable solvent ($CHCl_3$, acetone or ethyl acetate or their mixtures).

Preparation of Acetates

These were prepared by the acetic anhydride–pyridine method and isolated as usual. Solvent 3 was found to give good resolution in TLC; 25% H_2SO_4 spray was employed to locate the spots.

"*Dry Column*" *Chromatography*[16]

The crude pigment (50 mg) from the *Cupressus funebris* leaves was dissolved in pyridine (1 ml), silica gel (2 g) was added and mixed well; after the solution was adsorbed, the silica gel was dried at 120° for 2 hrs and then left in a vacuum desiccator over H_2SO_4 to remove all pyridine. The silica gel on which the pigment had been adsorbed was placed at the top of a column (10 cm × 1·5 cm) of dry silica gel. Solvent system 1 was allowed to percolate slowly into the column when the individual components of the pigment mixture separated as distinct yellow bands. These were eluted with the same solvent and collected. After the removal of the solvent under reduced pressure the pigments were crystallized from suitable solvents, e.g $CHCl_3$–MeOH or pyridine–MeOH mixtures. The pigments were checked for homogeneity by TLC and were identified by co-TLC and preparation of derivatives. The biflavones of the *C. funebris* leaves were eluted from the column in the following order: isocryptomerin, hinokiflavone, cupressuflavone and amentoflavone.

Acknowledgements—The authors thank Dr. N. Kawano for samples of isocryptomerin and its ethyl ether, Dr. P. V. Raman for help during the early stages of the investigation, Dr. P. N. Chatterjee and Mr. T. N. C. Vedantham for collection and Dr. Hardev Singh for identification of the plant materials.