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# BIFLAVONES OF THE SUBFAMILY CALLITROIDEAE, CUPRESSACEAE

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**Key Word Index**—Callitroideae; Cupressaceae; leafy twigs; chemotaxonomy; biflavones; amentoflavone; cupressuflavone; hinokiflavone.

**Abstract**—Twenty species, including representatives of all 11 genera of the Callitroideae, were examined for biflavonoid content of the leafy twigs. The major biflavonoids are based on amentoflavone, cupressuflavone and hinokiflavone. Their uneven distribution amongst the genera allows the distinction of five groups. These do not correlate strongly with currently recognized tribal groupings. The affinities of these genera are discussed.

## INTRODUCTION

The subfamily Callitroideae, according to Li [1], includes all the southern genera in the family, as well as the monotypic northern genus *Tetraclinus* Masters. Although de Laubenfels [2] has expressed doubt as to the basis of some recently defined genera, 10 southern genera are usually recognized [3–5]. Li divides them into two tribes: viz. Actinostrobeae containing *Actinostrobus* Miq., *Callitris* Vent. and *Fitzroya* Hook.; and Libocedreae, which contains *Neocallitropsis* Florin, *Widdringtonia* Endl., *Diselma* Hook. f., *Papuacedrus* Li, *Pilgerodendron* Florin, *Austrocedrus* Florin and Boutelje, and *Libocedrus* Endl. emend. Florin and Boutelje.

Previous studies on the occurrence of biflavones from the subfamily Callitroideae have been confined to a single genus, *Callitris* [6–9]. These indicate that *Callitris* contains a simple pattern of derivatives based on amentoflavone. The occurrence of hinokiflavone as a minor fraction has been reported in some studies [7, 9], but this could not be confirmed in others [6, 8]. Biflavones have previously been shown to be taxonomically useful, being generally uniform within species, but often highly variable between species or genera [6, 10–12]. It has been suggested that the affinities of the genera of this family, particularly between northern and southern Hemisphere genera, may be indicated by biflavonyl distribution [10, 6].

As part of a comprehensive study of the Cupressaceae, representatives of the Callitroideae were sampled to obtain the pattern of occurrence of biflavones amongst the genera.

## RESULTS

Investigations on many of the species were made on relatively small samples (20–50 g dry wt), but these proved adequate for the isolation of the major biflavones and their characterization by TLC and permethylation. Permethylations were carried out at three stages to check initial identifications against authentic markers, and to indicate the identity of parental biflavones in various samples. The crude extracts were permethylated to identify the parent biflavonyls present. This allowed the recognition of trace amounts of parent compounds, the derivatives of which could not be isolated in the normal way. Most BPF bands were permethylated to determine the parent biflavones present; often these bands contained derivatives of more than one parent biflavone. All bands were further refined on cellulose plates in BN, and the fractions identified using co-chromatography with authentic markers; a final permethylation was performed where sufficient material was available as confirmation of the parental biflavonyl structure. Permethyl ethers were identified by chromatographic comparisons with permethyl ethers obtained from authentic samples of biflavones, as well as by comparisons of UV fluorescence and UV spectra.

The biflavonyl patterns obtained for the Callitroid genera are given in Table 1.

## DISCUSSION

*Callitris*, with 15 species, is the largest Callitroid genus. Nine species have now been examined for biflavone content and only one species does not conform to the pattern reported by Gadek and Quinn [6]; viz. amentoflavone as the major band with possible minor bands of the 4‴- and 4‴,7″-methyl ethers. *C. neocaledonica* is exceptional in having major bands of all three biflavones, as well as the 4′-monomethyl ether. It is interesting to note that this species is also atypical of the genus in cone morphology. de Laubenfels [2] considers it a natural but specialized member of the genus, showing closest affinity to *C. sulcata*, the other New Caledonian species and the chemical evidence does not disagree with this view.

*Diselma* contains the richest array of biflavones in the Callitroideae, including trace amounts of a fourth parental biflavone detectable only by permethylation of the crude extract and tentatively identified as robustaflavone. This biflavone has previously been isolated from *Juniperus phoenica*, a member of the northern subfamily, Cupressoideae [13].

Four of the five species of *Widdringtonia* have been examined. A chemical discontinuity has been detected, with *W. dracomantana* and *W. juniperoides* containing 7″-monomethylhinokiflavone but not 4‴,7″-dimethylamentoflavone, while in *W. whytei* and *W. cupressoides* the situation is reversed. This difference coincides with a

Table 1. Occurrence and distribution of biflavones is the leaves of Callitroid species

| | | Biflavones | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Extracts | | | | | | | | Permethyl extract | | | | | |
| | Tribe/Genus/Species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | HAm | HCu | PH | PR | U1 | U2 |
| Act. | *Actinostrobus acuminatus* | + | t | — | — | — | — | — | — | + | — | — | — | — | — |
| Act. | *A. pyramidalis* | + | t | — | — | — | — | — | — | + | — | — | — | m | — |
| Act. | *Callitris macleayana* | + | t | — | — | — | — | — | — | + | — | — | — | — | — |
| Act. | *C. oblonga* | + | + | — | m | — | — | — | — | + | — | — | — | — | — |
| Act. | *C. sulcata* | + | m | — | t | — | — | — | — | + | — | — | — | — | — |
| Act. | *C. neocaledonica* | + | + | + | + | — | — | — | — | + | — | — | — | m | — |
| Lib. | *Neocallitropsis pancheri* | + | + | — | m | m | t | — | — | + | — | + | — | — | m |
| Lib. | *Papuacedrus papuana* | + | + | — | m | — | — | — | — | + | — | + | — | — | — |
| Lib. | *P. torricellensis* | + | + | — | — | t | — | — | — | + | — | + | — | — | — |
| Lib. | *Pilgerodendron uniferum* | + | + | — | — | t | — | — | — | + | — | + | — | — | — |
| Lib. | *Libocedrus yateensis* | + | m | — | — | — | — | — | — | + | — | m | — | — | — |
| Lib. | *L. plumosa* | + | m | — | — | — | — | — | — | + | — | m | — | — | — |
| Lib. | *Austrocedrus chilensis* | + | + | — | — | — | — | — | — | + | — | + | — | — | — |
| Act. | *Fitzroya cupressoides* | + | m | — | — | — | — | t | + | + | + | m | — | — | — |
| Lib. | *Widdringtonia dracomantana* | + | — | + | — | t | + | — | m | + | m | + | — | — | — |
| Lib. | *W. juniperoides* | + | t | + | — | t | + | — | + | + | + | + | — | — | — |
| Lib. | *W. whytei* | + | — | + | m | m | t | — | + | + | + | + | — | — | — |
| Lib. | *W. cupressoides* | + | m | + | + | t | — | — | + | + | + | + | — | — | — |
| Lib. | *Diselma archerii* | + | — | + | + | t | + | — | + | + | + | + | t | — | — |
| Tet. | *Tetraclinus articulata* | + | m | — | — | — | — | + | — | + | + | t | — | — | — |

Key: Act. = Actinostrobeae; Lib. = Libocedreae; Tet. = Tetraclineae. 1, Amentoflavone; 2, 4‴-monomethylamentoflavone; 3, 4′-monomethylamentoflavone; 4,4‴,7″-dimethylamentoflavone; 5, hinokiflavone; 6, 7″-monomethylhinokiflavone; 7, cupressuflavone; 8, 7,7″-dimethylcupressuflavone. HAm = Hexamethylamentoflavone; HCu = hexamethylcupressuflavone; PH = pentamethylhinokiflavone; PR = putative hexamethylrobustaflavone, U1 = unknown methyl ether 1; U2 = unknown methyl ether 2. + = Major band; m = minor band; t = trace detected TLC only.

morphological distinction noted in refs. [14, 15]. The first two species are characterized by 3–4 ovules per cone scale (cf. 6–10), up to 12 seeds per cone (cf. up to 30) and pollen sacs that are concealed within the male cone (cf. pollen sacs protruding from the cone). It will be interesting to see if this agreement between the chemical and morphological discontinuities is maintained when material of *W. schwarzii*, which belongs to the first group on morphological criteria, is available for chemical analysis.

While the separation of *Pilgerodendron* from *Libocedrus sensu lato* by Florin [16] has received general acceptance, de Laubenfels [2] has questioned Li's recognition of *Austrocedrus* and *Papuacedrus* as distinct genera. The only difference in biflavonyl pattern between these genera is the absence of hinokiflavone as a detectable band in both species of *Libocedrus* examined (Table 1), but minor amounts of hinokiflavone pentamethyl ether were detected in the crude extracts on permethylation. This homogeneity of the biflavonyl patterns certainly supports the recognition of these genera as constituting a closely allied group.

An examination of Table 1 shows that the major biflavonyls of the Callitroideae are derived from the same three parental biflavones as have been reported from members of the northern subfamily, Cupressoideae: viz. amentoflavone, hinokiflavone and cupressuflavone. As in the northern genera, however, they are not uniformly distributed. The following five generic groups can be recognized on the biflavonyl pattern.

(1) *Callitris* and *Actinostrobus*

Characterized by the presence of amentoflavone derivatives and the absence of detectable hinokiflavone derivatives. The only recent report of the occurrence of hinokiflavone in *Callitris* is for *C. glauca* R.Br [9], where it was detected as a minor component. Our own examination of Australian material of this species revealed no trace of hinokiflavone derivatives, or of its permethyl ether in the permethylated crude extract. This is in agreement with Prasad and Krishnamurti's observations on *C. rhomboidea* [8], as well as our own studies on seven other species. It can be concluded, therefore, that the occurrence of hinokiflavone in concentrations that can be detected by our survey methods is not a feature of *Callitris* species. This is in marked contrast to its reliable detection in all other Callitroid genera, except *Actinostrobus*, and indicates a clear genetic difference between these two groups of genera.

(2) *Neocallitropsis*

Characterized by major bands of amentoflavone and a monomethyl amentoflavone, and minor bands of hinokiflavone, a monomethyl hinokiflavone and a dimethyl amentoflavone. de Laubenfels [2] concluded that *Neocallitropsis* has an affinity with *Callitris*, more particularly with *C. neocaledonica*, on the basis of similarities in cone structure and intermediate leaves. Chemically, how-

ever, the two are clearly distinguished by the absence of detectable amounts of hinokiflavone derivatives in all the *Callitris* species examined, and the presence of an as yet undetermined compound as a minor component of the amentoflavone band of *Neocallitropsis* in BPF.

(3) *Libocedrus, Papuacedrus, Austrocedrus* and *Pilgerodendron*

Chemically a highly uniform group characterized by major amounts of amentoflavone and its 4‴-monomethyl ether, as well as trace amounts of hinokiflavone or its derivatives detectable by permethylation of the extract.

(4) *Fitzroya, Widdringtonia* and *Diselma*

Characterized by the presence of 7,7″-dimethylcupressuflavone, as well as major amounts of amentoflavone and its partial methyl ethers. This is a rather diverse group, both chemically and morphologically. The monotypic *Fitzroya* is distinguished by the absence of hinokiflavone derivatives (detectable only in the permethylated crude extract) and dimethyl ethers of amentoflavone, and has somewhat smaller amounts of the monomethylamentoflavone. de Laubenfels [17] has recently proposed a close relationship between *Fitzroya* and *Diselma* on morphological grounds. Boutelje [18] and Moseley [19] consider *Fitzroya* a distinctive genus, with wood and cone scale characters indicating a transitional position between northern and southern genera in the family. *Widdringtonia* has previously been allied with *Callitris* and *Actinostrobus* [2, 19, 20], but there is no evidence of such affinity on the basis of biflavonyl pattern. Heartwood chemistry [21] indicates *Widdringtonia* to be a distinctive genus having affinities with both northern and southern genera.

(5) *Tetraclinus*

This genus has amentoflavone and cupressuflavone as its major biflavonyls. It is distinguished from the previous group by the absence of dimethyl ethers. Hinokiflavone derivatives are present in the trace amounts detectable only by permethylation of the raw extract. Long considered distinct amongst the Cupressaceous genera [20], its chemical alliances would appear to be with the northern genera, where similar patterns have been recorded in *Cupressus* [10]. Heartwood chemistry also casts doubt on its placement in the Callitroideae and indicates affinities with northern genera [21].

The last two groups are clearly related by the presence of cupressuflavone or its derivatives, which must be regarded as a chemical specialization within the family [10, 22].

A comparison of the above generic grouping with those of Li [1] shows marked inconsistencies. Cupressuflavones are found in members of all three tribes of the Callitroideae, as well as in members of the other subfamily. Only the treatment of *Tetraclinis* as a highly distinctive genus assigned to its own tribe appears to be supported by the chemical data, though its placement with the southern genera in the Callitroideae is suspect.

Since Li's classification is based primarily on the analysis of cone-scale characters, it is possible that the taxonomic importance of this character has been overrated. Indeed, recent work by de Laubenfels [2, 17] has questioned the validity and importance of this character in assessing taxonomic relationships. A redefinition of the suprageneric taxa within the family as a whole must await a full reassessment of the morphological data.

## EXPERIMENTAL

Details of collections and voucher specimens are given in the Appendix. For each sample, the leaves and small branchlets were dried, crushed, and extracted in 70% EtOH for 48 hr, filtered, washed with petrol (bp 60–80°) if needed, and the residue dried. The residue was re-extracted in EtOH and the resulting extract was chromatographed on both thick and pre-coated Si gel plates developed in $C_6H_6$–pyridine–$HCO_2H$ (BPF) (100:20:7). Biflavones appeared as a number of dark, UV-absorbing bands, fluorescing yellow or orange on addition of $AlCl_3$. Each band was extracted individually and a further separation carried out on pre-coated cellulose plates developed in freshly prepared *n*-BuOH–2 N $NH_4OH$, [1:1 (upper layer)] (BN). Initial identifications were made in comparison with authentic markers in both solvents. Authentic markers of amentoflavone, 4‴-monomethylamentoflavone, 4‴,7″-dimethylamentoflavone, hinokiflavone and 7″-monomethylhinokiflavone were obtained from H. Geiger, whilst cupressuflavone, 4′-monomethylamentoflavone and 7,7″-dimethylcupressuflavone were isolated from *Araucaria bidwillii* and *A. cunninghamii* [23–26]. It was observed that bands composed of amentoflavone based derivatives fluoresced yellow when reacted with $AlCl_3$, hinokiflavone derivatives dark to bright yellow and cupressuflavone derivatives orange.

Permethylations of crude extracts, bands and purified compounds were carried out using $Me_2SO_4$ in dry boiling $Me_2CO$ and fused $K_2CO_3$. Permethyl ethers of amentoflavone, cupressuflavone and hinokiflavone were identified by permethylating

Table 2. Chromatographic and spectral data of permethylated parental biflavones

| Permethylated ethers | $R_f$s* | | UV fluorescence† | UV spectra ($\lambda_{max}^{EtOH}$) |
|---|---|---|---|---|
| | BPF | BPEFD | | |
| Amentoflavone | 0.37 | 0.40 | yellow | 267, 328 |
| Cupressuflavone | 0.41 | 0.45 | orange | 268, 332 |
| Compound 2 | 0.42 | 0.58 | light blue | 265, 323 |
| Compound 1 | 0.44 | 0.54 | white/yellow | 258, 335 |
| ?Robustaflavone (trace) | 0.50 | 0.68 | light blue | |
| Hinokiflavone | 0.53 | 0.75 | blue | 265, 323 |

*$R_f$ values are variable, but the relative positions of the permethyl ethers in each solvent are characteristic.
†As observed on plates run in BPF and dried in a hood for *ca* 0.5 hr.

authentic markers and comparing them in BPF and $C_6H_6$–pyridine–ethyl formate–dioxan (BPEFD), as well as UV fluorescence and UV spectral analysis, as documented in the lit. [26–29].

Two as yet unidentified compounds were extracted in association with other biflavonoid bands. Compound 1 was extracted from *Actinostrobus pyramidalis* and *Callitris neocaledonica*, compound 2 from *Neocallitropsis*. Both compounds were extracted in association with amentoflavone or its derivatives in BPF and BN and when permethylated gave the same fluorescent products identified in the permethylated crude extracts. Initial UV spectral analysis on these permethylated ethers, together with UV fluorescence and chromatographic analysis (see Table 2), indicates that these compounds do not align with any reported data for biflavones. Further work is proceeding.

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## APPENDIX

Location and collecting details of voucher specimens are given below. Abbreviations of herbaria follow *Index Herbariorum* [30].

*Actinostrobus acuminatus*. Parl. UNSW 11567, J. T. Waterhouse 8. viii. 1981, WA. *A. pyramidalis* Miq. UNSW Symon 1.ii.1982, cult. Waite Arboretum 632, SA. *Callitris macleayana* (F. Muell.) F. Muell. UNSW 12864, Gadek 12.iv.1982, cult. NSW. *C. oblonga* Rich. UNSW Symon 1.ii.1982, cult. Waite Arboretum 1225, SA. *C. neocaledonica* Dummer NSW de Laubenfels 8.x.1957, NC. *C. sulcata* (Parl.) Schlechter NSW 28864 Hotchkiss 15.iii.1954, NC. *Neocallitropsis pancheri* (Carriere)de Laubenfels CANB. Hartley 15068 23.xi.1979, NC. *Papuacedrus papuana* (F. Muell.)Li UNSW 4206 Quinn 24.vi.1974, PNG. *P. papuana* (F. Muell.)Li UNSW 4213 Quinn 24.vi.1974, PNG. *P. torricellensis* (Diels)Li NSW van Royen NGF 18250 6.ix.1963 PNG. *Tetraclinus articulata* (Vahl.)Masters UNSW Gadek 20.5.1981, cult. NSW. *Diselma archeri* Hooker fil. UNSW Gadek 15.9.1981, cult. UNSW. *Austrocedrus chilensis* (D. Don)Florin & Boutelje NSW de Barba 980 23.ii.1946, SAm. *Pilgerodendron uviferum* (D. Don)Florin NSW Sargent 21.i.1905, SAm. *Fitzroya cupressoides* (Mollina)Johnston NSW de Barba 1045 4.iii.1946, SAm. *Libocedrus yateensis* Guill. NSW de Laubenfels 4.xii.1957, NC. *L. plumosa* (D. Don)Sargent NSW Petrie vi.1910, NZ. *Widdringtonia dracomantana* Stapf NSW 989 vi.1920, SAf. *W. whytei* Rendle NSW Dorren-Smith 10.viii.1945, cult. *W. juniperioides* (L.)Endl. UNSW Symon 1.ii.1982, cult. Waite Arboretum 1275A, SA. *W. juniperioides* (L.)Endl. UNSW Symon 1.ii.1982, cult. Waite Arboretum 1231, SA. *W. cupressoides* (L.)Endl. UNSW Symon 1.ii.1982, cult. Waite Arboretum 1283A, SA.

Abbreviations: WA, Western Australia; SA, South Australia; NSW, New South Wales; NC, New Caledonia; PNG, Papua New Guinea; SAm, South America; SAf, South Africa.