Biflavanoids

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Chemical and Pharmacological Aspects

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CHAPTER 1

Structural Features and Occurrence of Biflavanoids

1.1 INTRODUCTION

Biflavanoids are the polyphenolic molecules gaining increasing recognition as modulators of physiological and pathological responses. Biflavanoids occur in many fruits, vegetables, and plants. The first biflavanoid, a biflavone, was isolated in 1929 by Furukawa who extracted the leaves of maidenhair tree, *Ginko biloba* L., and obtained a yellow pigment, which later proved to be a biflavanoid and was given the name ginkgetin (**1**; Fig. 1.1) [1]. However, with increasing research in the field of Natural Products a number of different bioflavonoids were discovered. Some important naturally occurring bioflavonoids include Amentoflavone (**2**), Isoginkgetin (**3**), Ochnaflavone (**4**), Morelloflavone (**5**), Hexamethylmorelloflavone (**6**), Robustaflavone (**7**), and Hinokiflavone (**8**) (Fig 1.1). However, all of the biflavanoids isolated and characterized still comprised of two identical or nonidentical flavanoid units joined in a symmetrical or unsymmetrical pattern through an alkyl- or an alkoxy-based linker of varying degree of length (Fig. 1.2). Each flavanoid subunit consists of a bicyclic ring system identified as rings A and B, while the unicyclic ring is named ring C. The two monomeric flavanoids are identified using Roman numerals I and II. The numbering in each subunit begins with the ring containing the oxygen atom. The variations possible in the parent flavanoid units, coupled with the large number of permutations possible in the position and nature of the interflavanoid linkage, introduce significant structural diversity in biflavanoids. This diversity is further amplified by variably positioned functional groups, for example, OH, MeO, C═O groups, or C═C bonds, and stereogenic centers on the flavanoid scaffold. In total, the class of biflavanoids represents a library of some 20,000 diverse molecules, each of which is capable of multiple H-bonding and hydrophobic interactions. Not all of these have been found to exist in nature as yet. However, in an age that values structural diversity, the theoretical library of biflavanoids spans a wide range of configurational and conformational space suggesting that the possibilities of interesting biological activity from

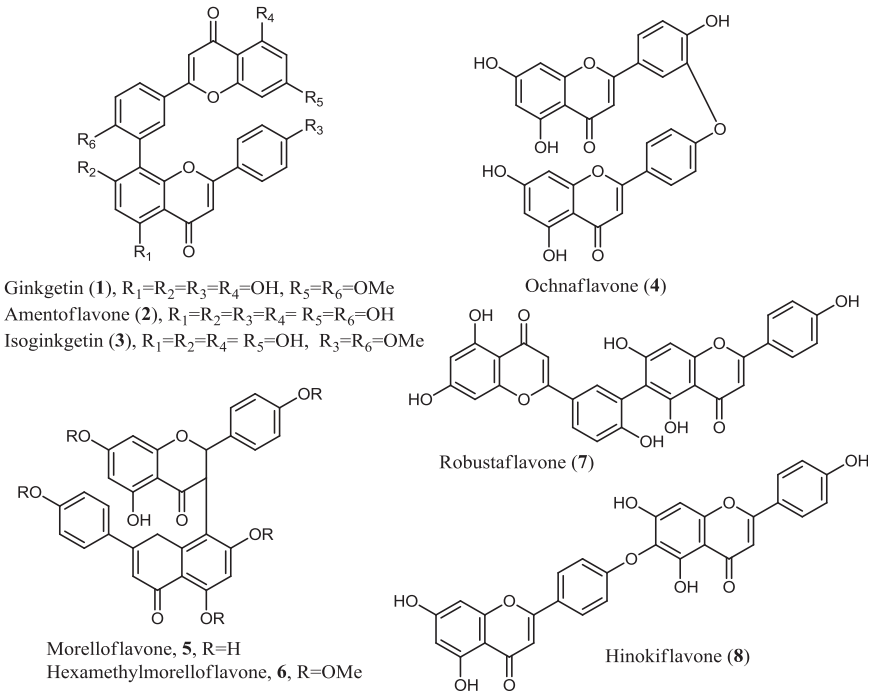

Figure 1.1 Structure of some common biflavanoids.

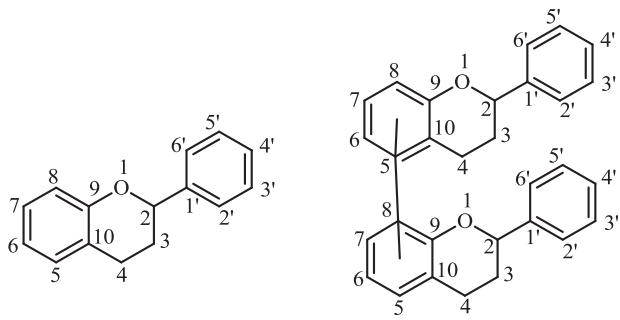

Figure 1.2 Basic structure of flavanoids and biflavanoids.

natural products are increasingly strong [2–15]. Structurally, biflavanoids are polyphenolic molecules.

1.2 NOMENCLATURE OF BIFLAVANOIDS

The rapid growth in the literature has led to several systems of naming these compounds in addition to the widespread use of their trivial

names to describe biflavanoids. However, Locksley has proposed several rules for naming the biflavanoids [16]. Locksley proposed that the generic name "biflavanoid" be used in place of biflavonyl to describe the family of flavanoid dimers. As per this nomenclature, the term biflavanoid has been adopted in preference to biflavanoid, as it more accurately reflects the saturated system as being the parent system. The ending "oid" may then be modified to cover specific types of homogeneous flavanoid dimers, such as biflavanone, biflavone, biflavan, and others, while, for mixed systems, the description "flavanone−flavone" should be used. This system generally follows the IUPAC recommendations. The unmodified monomer nomenclature is utilized as a generic term in the naming of dimeric, trimeric, tetrameric, etc., derivatives by insertion of the appropriate Greek prefixes, bi-, ter-, quarter-, etc., giving the biflavanoid, terflavanoid, quarterflavanoid etc. Locksley also standardized the nomenclature of the rings and the positions on rings. Each monomer unit is assigned a Roman numeral **I** and higher in a sequential manner. The intermonomer linkage is identified using a Roman numeral, which corresponds to the flavanoid unit, and an Arabic numeral, which corresponds to the position of the linkage. The two numerals for both the flavanoid monomers constituting the dimer are coupled with a hyphen and enclosed within square brackets. This represents the intermonomer linkage. The numbering of substituent groups on the monomeric units follows the IUPAC system for flavones, in which the three rings are referred to as A, B, and C (Fig. 1.2). For example, as per Locksley's system hexamethylmorelloflavone would be named as I-3′,II-3′,I-5,II-5,I-7-II-7-hexamethoxyflavone-[I-3,II-8]-flavone. Similarly, amentoflavone would be named I-4′,II-4′,I-5,II-5,I-7,II-7-hexahydroxy[I-3′,II-8]-biflavone and hinokiflavone, whose flavone units are linked through an O atom, would be named II-4′,I-5,II-5,I-7,II-7-pentahydroxy[I-4′-*O*-II-6]-biflavone. IUPAC has also devised its own system of nomenclature for biflavanoids. For example, hexamethylmorelloflavone as per IUPAC nomenclature is called as 5,7,5′,7′-tetramethoxy-2,2′-bis(4-methoxyphenyl)-2,3-dihydro[3,8′]bichromenyl-4,4′-dione while amentoflavone has been named as 8-[5-(5,7-dihydroxy-4-oxo-4*H*-chromen-2-yl)-2-hydroxy phenyl]-5,7-dihydroxy-2-(4-hydroxyphenyl) chromen-4-one. Likewise, hinokiflavone would be named as 6-[4-(5,7-dihydroxy-4-oxo-4*H*-chromen-2-yl)phenoxy]-5,7-dihydroxy-2-(4-hydroxy phenyl)chromen-4-one. This means that the difference between the Locksley and IUPAC nomenclature is the reference

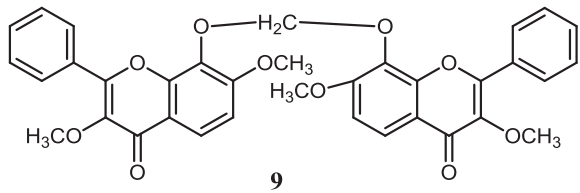

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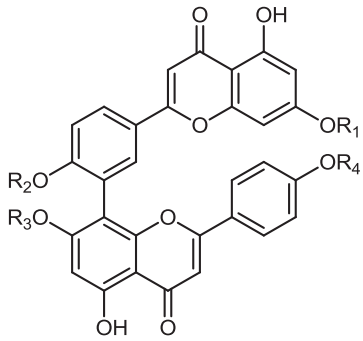
*Figure 1.3 Structure of oxyalkyl-linked biflavanoid (**9**).*

skeleton. Whereas the IUPAC system considers the majority of biflavanoids as derivatives of the chromene structure, the Locksley system uses the flavanoid structure. Thus, for oxyalkyl-linked biflavanoids, for example, compound **9** (Fig. 1.3), the IUPAC system has to change its reference skeleton, and this introduces considerable complexity in nomenclature. That is why scientists and researchers don't use these names but instead they use common names, for example, amentoflavone, cupressuflavone, and agathisflavone, which are easier. However, these names are limited because they contain no structural descriptors.

1.3 STRUCTURES OF BIFLAVANOIDS

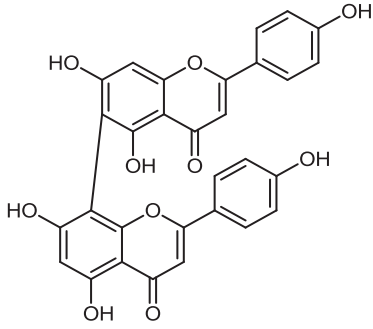
The majority of naturally occurring bioflavonoids are flavone and flavanone dimers with a simple 5,7,4′- or more rarely a 5,7,3′,4′-oxygenation pattern. In the majority of the cases the interflavanoid linkage is carbon−carbon, for example, in amentoflavone, with a few biflavanoids having carbon−oxygen−carbon linkage, as in hinokiflavone. The constituent monomers may be flavone−flavone, flavone−flavanone, or flavanone−flavanone. The hydroxyl groups may either be partially or fully methylated. Sixteen methylated derivatives of amentoflavone are known and many of these have isomeric structures. Rarely, other biflavanoids are possible, for example, based on two chalcone units [17] and one C-methylated biflavanoid, 7-*O*-methyl-6-C-methyl amentoflavone [18] has been found to date. Very recently two isoflavone−flavone dimers have been isolated from mosses [19]. Biflavanoids may be conveniently classified according to their interflavonoid linkage and basic flavone structure. The largest group has a 3′,8″ carbon−carbon bond and contains some 32 structures based on the parent compound amentoflavone (**2**) and its methyl ethers (**1,3, 10**−**15**) (Fig. 1.4).

Other groups include the 6,8″-agathisflavone (**16**) series with nine structures, the 8,8″-cupressuflavone (**17**) (Fig. 1.5) series with 14 compounds,



Ginkgetin (**1**), R_1=R_2=Me, R_3=R_4= H
Amentoflavone (**2**), R_1=R_2=R_3=R_4= H
Isoginkgetin (**3**), R_2=R_4= Me, R_1= R_3= H
Sequoiaflavone, (**10**), R_1= Me, R_2=R_3=R_4= H
Bilobetin (**11**), R_2= Me, R_1=R_3=R_4= H
Sotetsuflavone (**12**), R_3= Me, R_1=R_2=R_4= H
Podocarpusflavone A (**13**), R_4= Me, R_1=R_2=R_3= H
Sciadopitysin (**14**), R_1=R_2=R_4= Me, R_3= H
Kayaflavone (**15**), R_2=R_3=R_4= Me, R_1= H

Figure 1.4 Biflavanoids of the amentoflavone series.



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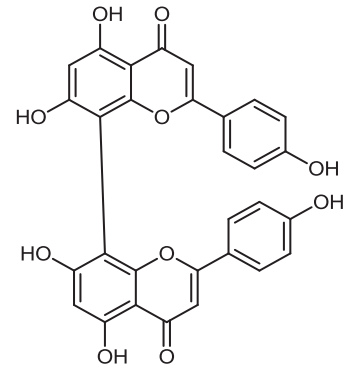

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Figure 1.5 Structures of agathisflavone and cupressoflavone.

and the 6,3″-robustaflavone (**7**) series with five structures. The largest group of bioflavonoids with a carbon−oxygen−carbon interflavonoid linkage is based on hinokiflavone (**8**) and there are some 11 structures, all with a 6,4‴ linkage. The known biflavanoid series are summarized in Table 1.1.

1.4 NATURAL DISTRIBUTION OF BIFLAVANOIDS

Biflavanoids are generally distributed as characteristic compounds of gymnosperms, psilotales, and selaginellas with a very small distribution in angiosperms. In addition, they have been also reported in two ferns and a few moss species. The biflavanoids have been reported in Bryales in bryophytes, Psilotales, Isoetales, Lycopodiales, Slagenellales, and Filicales. Similarly, among the gymnosperms the

Table 1.1 A Summary of Known Flavanoids

Linkage	Parent Compound	No. of Structures	Monomer Type[a]
C−C linked			
5,7,4′-Oxygenation pattern			
3,3″	Biapigenin	1	A−A
3,3″	Chamaejasmin	12	B−B
3,8″	Garcinia biflavanoids	15	A−A, B−A, B−B
3,8″	Compound E	1	D−C
3,8″	2Fl	1	D−D
3,8″	Zeyherin	1	D−E
3,3‴	Taiwania flavone	4	A−A
6,6″	Succedaneaflavone	4	A−A, B−B
6,8″	Agathisflavone (**16**)	9	A−A, B−A, A−B
8,8″	Cupressuflavone (**17**)	14	A−A, B−A, A−B
6,3″	Robustaflavone (**7**)	6	A−A, B−B
3′,6″	Hegoflavone	6	A−A, B−A
3′,8″	Amentoflavone (**2**)	32	A−A, B−A, B−A
3,3‴	Biapigenin hexamethyl ether	1	A−A
Mixed 5,7,4′ and 5,7,3′,4′ Oxygenation patterns			
3,8‴	Morelloflavone	1	B−A
3′,6″	Brayoflavone	1	F−A
3′,8″	Heterobryoflavone	1	F−A
3′,8″	Semecarpuflavanone	2	B−B
5,7,3′,4′-Oxygenation pattern			
5,6″	Biluteolin	2	A−A, A−B
5,8″	Biluteolin	1	A−A
2′,6″	Biluteolin	2	A−A, A−B
3′,8″	Strychnobiflavone	3	A−A
3′,8″	Galluflavanone	1	B−B
Ether linked			
6,4‴	Hinokiflavone (**5**)	11	A−A, A−B
6,4‴	Occidentoside	1	B−C
4′,8″	Pentamethoxy biflavonyl ether	1	A−A
3′,4‴	Ochnaflavone	5	A−A

[a] *A, flavone; B, flavanone; C, chalcone; D, dihydrochalcone; E, hydrated aurone; F, isoflavone.*

biflavanoids have been reported from orders which include Cycadales, Ginkgoales, Taxales, Gnetales, Coniferales, etc. Among the Cycadales, the monotypic Stangeriaceae does not contain biflavanoids. The family Cycadaceae of Cycadales is characterized by the presence of both the amentoflavone and hinokaflavone series. In Zamiaceae, amentoflavone derivatives are the major biflavanoid constituents known to date. In Coniferales the biflavanoids in leaves are the major constituents. The family Podocarpaceae of Coniferales is characterized by the presence of amentoflavone and its methyl esters with some abundance of hinokiflavone and its methyl esters. Similarly, in Taxodiaceae both the amentoflavone and hinokiflavone derivatives have been reported to be present, whereas in monotypic Cephalotaxaceae only the amentoflavone series is reported. In Cupressaceae the amentoflavone derivatives are found abundantly with robustaflovone as a trace constituent. In this family the hinokiflavone and cupressoflavone show a rather disjunct distribution, which correlates quite well with generic boundaries.

Biflavanoids are still comparatively uncommon in the angiosperms, having been recorded in 34 genera from 16 families. Whereas amentoflvone is widely spread in the angiosperms, hinokiflavone has been found in only certain families, such as Casuarinaceae, Anacardiaceae, and Iridaceae. Agathisaflavone methyl ethers appear to be good indicators of affinity within the Anacardaceae, however agathisaflavone has been found to be in Rosiflorae as well. Tetrahydrobustaflavone has been found in Anacardiaceae, whereas cupressuflavone occurs in the Clusiaceae and Casuarinaceae. While biflavanoids have been found in various dicotyledonous species they have been found in only a very few monocotyledonous species, but there is provisional evidence that they may also be present in further members of the Iridaceae and Velloziaceae.

1.5 CONCLUSION

In conclusion this chapter highlights the basic aspects of biflavanoids with particular reference to their occurrence, isolation, nomenclature, structure, and distribution.

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CHAPTER 2

Isolation and Identification of Biflavanoids

2.1 INTRODUCTION

Most of available methods for the separation and identification of flavanoids are also employed in the determination of biflavanoids. A few of the most commonly used techniques include paper chromatography (PC), thin-layer chromatography (TLC), column chromatography (CC), and UV spectroscopy, mass spectroscopy (MS), and NMR spectroscopy studies. High-pressure liquid chromatography (HPLC), although a very strong technique, has, however, not been used so much in the determination of biflavanoids.

2.2 SEPARATION AND PURIFICATION

Generally, biflavanoids are soluble in all solvents except less polar aliphatic hydrocarbons, for example, hexane, which are therefore useful for defatting crude extracts. The solubility of a biflavanoid depends very much on its structure, especially the degree of methylation and the nature of the plant material in which it is present. For example, amentoflavone methyl ethers can be extracted easily into methylene dichloride, benzene, and trichloroethylene. Chloroform has been used successfully to isolate the *Garcinia* biflavanoids. However, the highly hydroxylated biflavanoids, which include amentaflovone, are extracted with polar solvents like absolute or aqueous methanol or ethanol. It is very difficult to extract the biflavanoids from mosses because these compounds are located within the cell walls of these plant species. The best way to extract from them is to extend the time of extraction using a polar solvent like methanol with the addition of some 10−20% water for efficient extraction.

Basic methods of purification include the distribution of the crude extract between two immiscible solvents. For example, the less polar fatty compounds are separated by distribution between light petroleum and dimethyl formamide, and the water soluble contaminants are

removed by distribution between ethyl methyl ketone and water. However, the final process of purification depends upon the nature and amount of plant material. Preparative TLC and PC is used for small plant extracts, while for larger quantities of extracts CC is used very often.

2.2.1 Preparative PC and TLC

Preparative PC on Whatmann 3 MM paper run in BAW (butanol: acetic acid:water, 4:1:5, top layer) is a preliminary separation of the biflavanoid fraction from the crude extracts. Here the biflavanoids run up to the top of the chromatogram and can be seen as a dark absorbing band. The band is then cut and eluted with methanol. However, the purification of pure individual biflavanoids is then carried out by using preparative TLC. Preparative TLC is generally carried out on glass plates. Commercially available preparative TLC thinner plates (thickness = 0.25 mm) and thicker plates may be used for small and larger quantities of extracts, respectively. However, an increase in thickness leads to a decrease in resolution. The choice of solvents used for their separation have been listed in Table 2.1.

Table 2.1 Solvent System Used for the Isolation of Bioflavonoids Using Preparative Thin-Layer Chromatography

Support	Solvent System	Biflavanoid	References
Silica gel	$CHCl_3$:MeOH (90:10)	Biflavones and 2,3-dihydrobiflavones	[1]
	$CHCl_3$:MeOH (95:5)	Permethylated biflavanoids	[1]
	$CHCl_3$:MeOH (5:1)	Biflavanoids	[2]
	$CHCl_3$:Me_2CO:HCOOH (9:2:1)	Biflavanoids	[3]
	Tol:HCO_2Et:HCOOH (5:4:1) TEF	Biflavones and their partial methyl ethers	[4]
	Tol:Pyridine:HCOOH (100:20:7) TPF	Amentoflavone	[5]
	Tol:Pyridine:HOAc (10:1:1)	Biflavones and their partial methyl ethers by multiple development	[6]
	Tol:DMF:HOAc (10:1:1)		
	C_6H_6:Pyridine:HCOOH (36:9:5) BPF1	Partial methyl ether of amentoflavone, agathisflavone, and cupressuflavone	[7]
	C_6H_6:Pyridine:HCOOH (100:20:7) BPF2	Biflavone and their partial methyl ethers	[4, 8–10]
	C_6H_6:Pyridine:HCOOH (100:15:5) BPF3	Biflavone and their partial methyl ethers	[4]

Among the chloroform-based solvents used for the preparative TLC are those containing methanol up to 20%. The solvents with high methanol in chloroform are used for the separation of polar biflavanones and those with lower concentration of methanol are used for the separation of permethylated biflavanoids. Among the benzene-based or toluene-based solvents, toluene:ethyl formate: formic acid (5:4:1), abbreviated as TEF, was first used for analytical TLC of biflavanoids in conifers [6]. However, Quinn and coworkers have used it for semipreparative TLC after preliminary isolation of the biflavanoid fraction using PC in a butanol:acetic acid:water system. Natarajan et al. [6] isolated biflavones and their methyl esters from Cupressaceae by means of multiple developments in toluene: pyridine:acetic acid (10:1:1) and toluene:dimethylformaamide:acetic acid (10:1:1). These solvents may have been superseded but multiple developments still proves to be a useful tool for the separation of closely related constituents.

A new solvent system has been devised for the silica gel thin-layer chromatographic separation of biflavanoids from *Rhus succedanea* and *Garcinia multiflora*. This solvent system has permitted the complete separation of amentoflavone, cupressuflavone, and agathisflavone, and consists of C_6H_6:pyridine:HCO_2H (40:10:2) in a homogeneous solution. It was developed and used by Lin et al. [11].

2.2.2 Column Chromatography

CC is a useful technique for the separation of biflavanoids on a larger scale. Although many stationary phases have been used, it appears that silica gel, polyamide, and sephadex LH 20 have been more commonly and successfully utilized for the separation of biflavanoids using CC. A few of the examples citing the isolation of biflavanoids using CC are highlighted in Table 2.2. From Table 2.2 it can be seen that most commonly silica gel and polyamide have been used for the isolation of biflavanoids. Polyamide has a much greater capacity than silica gel and cellulose and is complementary to PC in that a different adsorbent and solvents are used. Polyamide 6, Polyclar AT, and Polyclar AT NE 62466 have all been used in the separation of apigenin-based biflavones and their methyl ethers, biluteolins, and isoflavone-flavone dimers. Sephadex LH 20 mainly separates mixtures on the basis of molecular size and is intended for use with polar organic solvents. It is helpful for both the cleaning up of crude

Table 2.2 Examples of Biflavanoid Isolations Using Column Chromatography

Column Support	Application Solvent	Composition and Order of Eluents	Biflavanoids	References
Silica gel (100−200 mesh)	Me_2CO (after Ppt of Pb salt)	$CHC1_3$, $CHCl_3$:Me_2CO (7:2) $CHCl_3$:Me_2CO (3:l), $CHCl_3$:Me_2CO(5:2), $CHCl_3$-Me_2CO (2:l), Me_2CO, prep TLC with $CHC1_3$:MeOH (5:1), CC $CHCl_3$:Me_2CO (1:1)	Jeediflavanone (biflavanone)	[2]
Silica gel	Et_2O (from 80% MeOH)	$CHCl_3$, $CHCl_3$-MeOH, mixtures of increasing polarity. $CHCl_3$:MeOH (19:1) gave the biflavanone	Chamaejasmin (Biflavanone)	[12]
Silica gel	Et_2O (from 80% MeOH and Ac_2O)	$CHCl_3$:MeOH (24:1)	Morelloflavone and its 4″ monomethyl ether (dihydrobiflavones)	[13]
Silica gel 60-PF (60 mesh)	EtOH partitioned between $CHCl_3$ and H_2O interface residue	$CHCl_3$:MeOH (9:1)	3′,8″-Binaringenin Amentoflavone	[14]
Silica gel 60 Merck (mesh 70−230)	EtOAc	DCM:MeOH (14:9, sat. with H_2O), $CHCl_3$:MeOH:H_2O, (64:50:10)-*prep*. TLC.	Biflavanonols	[3]
Silica gel BDH (60-120 mesh)	EtOAc	C_6H_6:$CHCl_3$ (1:3)	5,3″,7″-triOH-7,4′,4‴-triOMe-(3′6″)-biflavone	[15]
Silica gel	MeOH (from Me_2CO and EtOAc)	Petrol (40-600) C_6H_6, EtOAc Me_2CO	7″-Methyl tetrahydroamentoflavone (biflavanone)	[16]
Silica gel	Me_2CO	Set with petrol, C_6H_6, EtOAc (9:1), C_6H_6- EtOAc (8:2)	Dihydrobiflavones methylated biflavones	[17]
Polyclar AT	95% EtOH and/or MeOH:pyridine (9:1)	$CHCl_3$:MeOH:MeCOEt (4:2:1)	Biflavones and their methyl ethers	[18]
Polyclar ATNE 62466	Me_2CO	CH_2C1_2 increasing amounts of MeOH, CH_2Cl_2:MeOH (4:6)	4′,4‴-Dimethylcupressuflavone (bimethylbiflavone)	[19]
Polyamide 6[a]	80% EtOH	Me_2CO:H_2O (9:1) with increasing amounts of H_2O,Me_2CO:H_2O (1:4)	5′,6″-Biluteolin	[20]
Polyamide[b]	MeOH (from 80% EtOH)	EtOAc:MeCOEt:HOAc:H_2O (5:3:1:1)	Bryoflavone, heterobryoflavone (isoflavone−flavone dimers)	[21]
Polyamide 6[c]	Lower phase of DMF:H_2O:Et_2O (4:1:8)	Me_2CO:H_2O (4:1) increasing amounts of H_2O to Me_2CO:H_2O (1:4)	Biluteolins, 5′-Hydroxyamentoflavone	[22]

[a] *Followed by final purification on Sephadex LH-20 using Me_2CO:MeOH:H_2O (2:1:1) and MeOH:H_2O (8:2).*
[b] *Preliminary clean-up with Sephadex LH-20 with MeOH as eluent and final purification on Sephadex LH-20 with MeOH:Me_2CO:H_20 (8:1:1).*
[c] *Final purification on Sephadex LH-20 with Me_2CO:MeOH:H_2O (2:1:1).*

extracts as well as the purification of individual biflavanoids. The choice of solvents depends upon the TLC profiling. Most of the methods involve the use of a single solvent or a mixture of solvents, with the less polar solvent being used first, which is followed by the use of a more polar solvent or a solvent mixture. As in TLC the solvents used in CC are chloroform-based or benzene-based. Thus chloroform and chloroform:methanol mixtures have been used in the isolation of biflavones, biflavanones, dihydrochalcones, etc. For example, in the isolation of jeediflavanone, Murthy [2] used a complex sequence starting with chloroform with increasing amounts of acetone to pure acetone followed by PTL on silica gel in chloroform:methanol (5:1) and finally CC in chloroform:acetone (1:1). In contrast, Chatterjee et al. [15] employed a single solvent mixture of benzene:chloroform (1:3) to separate a trimethoxy biflavone. However, Sonnenbichler et al. [3] used quite a different sequence of solvents, dichloromethane:methanol (14:9, saturated with water) followed by chloroform:methanol:water (64:50:10) and this was completed by their final separation using PTL.

2.2.3 Droplet Counter-Current Chromatography

This technique has, however, not been used in the isolation of biflavanoids except for the initial separation of biluteolins from the combined methanol acetone extract of the moss *Dicranoloma robustum* by Markham et al. [23]. Droplet counter-current chromatography (DCCC) of the extract, redissolved in a mixture of the stationary and mobile phases, was carried out in the descending mode using chloroform: rt-butanol:methanol:water (10:1:10:6), monitored at 340 nm with a flow rate of 40 mL/h. This fraction was followed by large-scale one-dimensional paper chromatography (1 DPC) in 50% acetic acid and final purification by HPLC. Kapadia et al. [24] reported the separation of constituents from the defatted seeds of *Garcinia kola* using the high-speed counter-current chromatography technique. Using this technique with the solvent system *n*-hexane:ethyl-acetate:methanol:water (1:4:2.5:2.5) upper phase as the stationary phase and the lower phase as the mobile phase, the ethyl acetate extract provided seven products. Earlier biflavanoid separation of *G. kola* has been reported using DCCC using CHCl:MeOH:water as the solvent system (the more polar layer as the mobile phase) [25]. However, the separation of three products, in unspecified amounts has been reported.

2.2.4 High-Pressure Liquid Chromatography

Although HPLC is a very robust and solid technique used for separating the flavanoid monomers, so far it has been used in only a very few studies of biflavanoids. Thus, Briancon-Scheid et al. [26] obtained a fair separation of purified amentoflavone-type biflavones from *Ginkgo biloba* using a C_{18}-column and a gradient system using the solvents A, 5% acetic acid in acetonitrile, and B, 5% acetic acid in water. However, a better separation was achieved by the same authors [27] using a Lichrosorb diol column and a ternary elution system of solvent A, hexane:chloroform (25:75), and solvent B, tetrahydrofuran. Pietta et al. [28] have also obtained sharp separations of *Ginkgo* biflavones using tetrahydrofuran:propanol:water (21:10:69) isocratically. Gadek [29] used a Lichrosorb diol column to distinguish amentoflavone, hinokiflavone, and their partial methyl ethers using an isocratic elution with a 9:1 mixture of chloroform and tetrahydrofuran. However, this method did not separate all the trimethyl and dimethyl ethers of amentoflavone, hinokiflavone, and cupressuflavone and it is very difficult to resolve mixtures based on two or more parental structures. Markham et al. [23] employed a C_{18}-Column fitted with an RP-18 precolumn and the solvents A, methanol, and B, 3% formic acid in water, for separating biuteolins. Permethylated biflavone mixtures have been separated successfully using HPLC on silica gel columns eluting with either: (1) tetrahydrofuran:chloroform (1:1) linearly to 100:0 over 15 min at a flow rate of 2 mL/min; or (2) 100% tetrahydrofuran at a flow rate of 2.5 mL/min. A few HPLC systems that have been used in the isolation of bioflavonoids are shown in Table 2.3.

2.3 IDENTIFICATION

Various methods of identification of biflavanoids are described in the literature. These include co-chromatography, which involves the comparison with the known available authentic markers, UV−visible spectroscopy, NMR analysis, MS data, and chemical derivatization.

2.3.1 UV Spectroscopy

UV spectroscopy of biflavanoids resembles closely its monomeric forms. Table 2.4 gives the UV data of some known biflavanoids. Spectral data generally helps in identifying the nature of the monomeric units in biflavanoids as well as the position of the *O*-methyl substituents. The ratio of the extinction coefficient of the two main spectral bands is a diagnostic feature

Table 2.3 Some HPLC Systems Used for Separating or Purifying Biflavanoids

Solvents						
Column	**A**	**B**	**Elution Profile**	**Flow Rate (mL/min)**	**Detection**	**References**
C-18	5% AcOH in ACN	5% AcOH in H_2O	0–4 min, 60% A and 40% B, Isocratic elution 4–14 min, 60–75% A, Linear 14–24 min, 75% A, Isocratic	1.5	330	[26]
Hibar Lichrosorb Diol	THF	$CHCl_3$	0–4 min, 0.1–5% A, Linear 4–10 min, 5–50% A, Linear 10–18 min, 50% A and 50% B, Isocratic	1	330	[26]
Hibar Lichrosorb Diol	Hex: $CHCl_3$ (25:75)	THF	0–15 min, 0.1% A and 99.9% B, Isocratic 15–45 min, 0.1–50% A, Isocratic 45–65 min, 50–75% A, Linear 65–75 min, 75–25% B, Isocratic	1	330	[27]
Hibar Lichrosorb Diol	$CHCl_3$	THF	0–25 min, 90% A and 10% B, Isocratic	1	320	[28]
Nova pak C_{18}	THF	Propanol: H_2O (10:69)	0–15 min, 21% A and 79% B, Isocratic	1	270	[29]
C_{18}	MeOH	3% HCOOH in H_2O	0–25 min, 35–80% A, Linear	Not given	356	[23]

that distinguishes the monomeric units from their dimers. In biflavones the long wavelength band at around 330 nm is less intense than the short wavelength band at around 270 nm, that is, it has 86–97% intensity. However, in the case of the corresponding monomeric units the situation is quite the reverse. The long/short wavelength band varies from 112% to 122%. This is true of biflavones based on amentoflavone but has not been tested yet with other interflavanoid linkages. Spectral shift reagents can be used for biflavanoids as they can be used for the monomeric flavones and flavanones. Within a given biflavanoid series, measurement of neutral spectra and shifts can be helpful in assigning structures although there can occasionally be problems in interpretation because of the overlapping effect of monomeric flavanoid moieties present. It is apparent that

Table 2.4 UV Spectral Data of Some Known Biflavanoids

Biflavanoid	Neutral Maxima	Alkaline Maxima[a]
Amentoflavone (3′,8″-linked)[b]	268, 292sh, 336	273, 292sh, 380 (III)
4′-ME (Bilobetin)	275, 327	280, 300sh, 375 (DII)
7″-ME (Sotetsuflavone)	274, 288sh, 334	276, 296sh, 384 (III)
7,4-DiME (Ginkgetin)	268, 328	280, 325sh, 382 (DII)
4′,4‴-DiME (Isoginkgetin)	271, 328	278, 297sh, 367 (DII)
7″,4′,4‴-TriME (Kayaflavone)	270, 326	279, 295sh, 372 (DII)
7,4′,4‴-TriME (Sciadopitysin)	268, 326	280, 352 (DII)
7,7″,4′,4‴-TetraME	246sh, 272, 328	246sh, 272, 328 (–)
Hinokiflavone (O-linked)	268, 336 (low A)	268, 390 (DII)
Cupressuflavone (8,8″-linked)	272, 284sh, 330	275, 382 (III)
2,3-Dihydroamentoflavone	286, 326sh	292sh, 323, 396 (III)
Morelloflavone (Ap-Ludimer)[c]	260sh, 276sh, 284, 336	282, 322, 408 (–)

[a]sh, Shoulder, III, increase in intensity, DII, decrease in intensity, (–), no change.
[b]Amentoflavone gives an $AlC1_3$ spectrum with maxima at 280, 300sh, 349 and 399 nm and the methyl ethers respond similarly.
[c]NaOAc/borate spectral maxima are at 272, 285sh, and 372 nm. None of the other bioflavonoids listed here gives a positive borate shift.

methylation of the B-ring either at the 4′ or 4″-positions causes a hypsochromic effect in the long wavelength band between 3 and 10 nm. 4′-Methylation also causes a decrease in the intensity of the long band in the presence of alkali. In addition, increasing the methylation of amentoflavone reduces the size of the alkaline shift in this band. UV can also be useful for recognizing which monomeric units are linked together in a given biflavanoid nucleus. The flavone-flavanone dimers are clearly different in the neutral spectra since the long wavelength band is reduced in intensity. Thin-layer chromatography data coupled with UV−visible spectra has been used to differentiate between 19 biflavanoids, which are unsubstituted, partly methylated, or fully methylated [30]. Amentoflavone, hinokiflavone, cupressuflavone, agathisflavone, morelloflavone, and their derivatives have been fully studied. The spectra were recorded in the presence of shift reagents, including NaOMe, NaOAc, NaOAc−H_3BO_3, $AlCl_3$, and $AlCl_3$−HCl.

2.3.2 Mass Spectrometry

The mass spectra of both O-linked and C-linked bioflavonoids have been recorded mainly as their permethyl derivatives [31] and strong molecular ions can be observed. In general, the two flavanoid units of

C−C linked bioflavonoids each produce fragments by some of the pathways which are well defined for the corresponding monomeric compounds. Thus some A-ring and B-ring fragments are exactly the same as those observed for monoflavonoids, while others are typical A-ring and B-ring fragments except that they have intact flavanoid skeletons attached. Amentoflavone permethylether (MW 622), for example, produces a fragment of MW 311 based on the A-ring and B-ring attached in the middle of the molecule through the 3−8″ link and such a fragment could be considered to be a characteristic feature of the mass spectrometry of biflavanoids. The O-linked bioflavonoids tend to undergo fission on both sides of the O-linkage and hence four different monomeric units can be detected. Hinokiflavone permethylether (MW 608), for example, produces fragments at 327, 311, 297, and 281, all of which are of comparable intensity. These four fragments then undergo further fragmentation along predictable pathways. More recently, with the advent of fast atom bombardment (FAB)-MS and related techniques, it has been possible to determine the mass spectra without prior derivatization. Intense molecular ions can be obtained when using the negative mode. Fragmentation occurs during FAB-MS but not all the fragments can be easily assigned. With hinokiflavone, cleavage at the ether bridge gives predictable ions at 283, 281, 255, and 253, but other prominent ions at 355 (M-183) and 183 (M-355) appear to be fragments produced by cleavage of one of the aromatic rings [32]. Field desorption (FD)-MS also gives good molecular ions without derivatization [21]. This technique yields only the molecular ion and a few thermal fragments. Biflavanoids in which the *ortho* positions on either side of the interflavonoid link are occupied by hydroxyl groups (eg, agathisflavone) may lose water and show a $[M\text{-}18]^+$ fragment. Biflavanoids with flavanone residues may lose phloroglucinol and give an $[M\text{-}126]^+$ ion [33]. The most useful structural information now available from both FAB-MS and FD-MS is the determination of molecular weight; this can be measured on samples as small as 0.5 mg with considerable reliability.

2.3.3 NMR Spectroscopy

Proton NMR measurements have now been recorded for many biflavanoids, usually either in DMSO-d_6 or as the trimethyl silyl ethers in $CC1_4$ [31]. In DMSO-d_6, the spectra can occasionally be misleading since the exact position of the signals depends on the concentration and temperature and on whether traces of water are

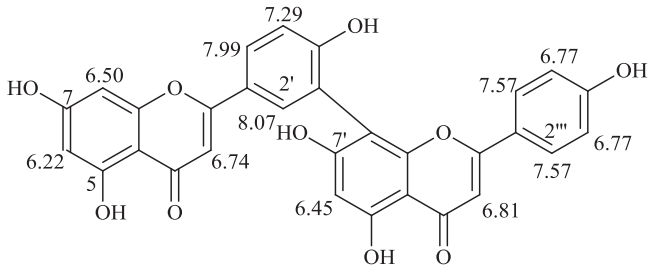


Figure 2.1 Proton NMR of amentoflavone in ppm measured in DMSO-d_6 *at 400 MHz.*

present. Typical results are illustrated in Fig. 2.1 for amentoflavone, based on the data of Geiger et al. [22]. This shows that the carbon–carbon interflavonoid linkage causes shifts in the proton signals of the two benzene rings involved. Proton NMR has in fact been mostly used to decide whether in a given case the C-6 or C-8 is involved in the interflavonoid link. This procedure requires the determination of benzene-induced methoxyl shifts, which occur only if one position ortho to a given methyl group is unsubstituted. Measurement is made on the fully methylated, or other substituted, biflavanoid, first in $CDC1_3$ and then in benzene, and the benzene-induced shift of a given methoxyl recorded. This method clearly separates the 6,8-linked agathisflavone series from the 8,8-linked cupressuflavone derivatives. The location of the interflavonoid link can also be determined by using the lanthanide shift reagent, $Eu(fod)_3$, instead [34]. Proton NMR measurements have also been used to determine the position of methyl substituents in the amentoflavone series, after acetylation of the remaining free phenolic groups [17]. Recently, 500-MHz proton NMR spectroscopy and circular dichroism have been applied to the determination of absolute configuration of two hydroxyl biflavanonols from *Garcinia cola* [3].

Carbon13-NMR spectroscopy was first applied successfully to monomeric flavanoids [35]. Some of the first studies of bioflavonoids were those of Chari et al. [36] and these have recently been extended by Markham et al. [37]. The latter authors measured the spectra of 13 biflavanoids at 20 MHz on 14–20 mg samples dissolved in DMSO-d_6. The spectral data for amentoflavone is shown in Fig. 2.2. It should be noted that one or two of the signal assignments may be interchangeable.

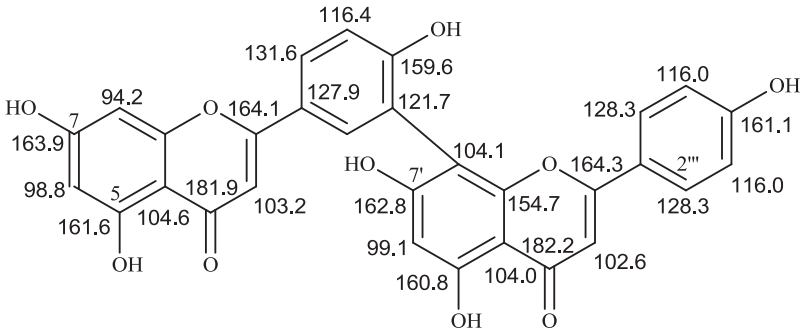
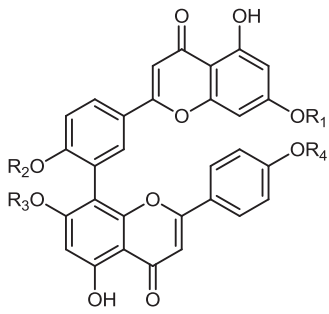


Figure 2.2 ^{13}*C-NMR signals for amentoflavone, in ppm, measured in DMSO-*d_6 *at 20 MHz.*



Amentoflavone, R_1=R_2=R_3=R_4= H
Bilobetin, R_2= Me, R_1=R_3=R_4= H
Podocarpusflavone A (13), R_4= Me, R_1=R_2=R_3= H
Isoginkgetin, R_2=R_4= Me, R_1= R_3= H
Ginkgetin, R_1=R_2=Me, R_3=R_4= H
Tetramethyl amentoflavone, R_1=R_2=R_3=R_4= Me
Robustoflavone, Same as amentoflavone, but with 3'—6'' interflavonoid link
Dihydroamentoflavone, R_1=R_2=R_3=R_4= H

Figure 2.3 Amentoflavone series.

The opportunity to record a range of ^{13}C NMR spectra on a series of related biflavanoids of amentoflavone series has recently been published for some biflavanoids [37]. This data relates the spectra structure correlations of both the amentoflavone series (Fig. 2.3) of natural compounds.

The ^{13}C NMR of amentoflavone has been assigned previously and here again these have been adopted except for those of I-1 and II-1‴. The assignments have been reversed for these two carbon atoms in order to accommodate the spectra of bilobetin and podocarpusflavone A. Assignments for the spectra of amentoflavone methyl ethers follow logically from the amentoflavone assignments, as does that of 2,3-dihydroamentoflavone when the spectrum of naringenin is a reference standard. However, there are certain changes in the assignments of spectral signals by Markham et al. [37] over those of the Chari et al. [36].

The data that has been presented in Table 2.5 for the amentoflavone series of biflavanoids permits the calculation of the following

Table 2.5 ^{13}C NMR of Amentoflavone, Its Derivatives and Reference Compounds

	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′
Amentoflavone															
I	164.1[a]	103.2[b]	181.9[c]	161.6	98.8[d]	163.9[e]	94.2	157.6	104.0	120.3	127.9	121.7[f]	159.6	116.4	131.6
II	164.3[a]	102.8[b]	182.2[c]	160.8	99.1[d]	161.9[e]	104.1	154.7	104.0	121.4[f]	128.3	116.0	161.1	116.0	128.3
Bilobetin															
I	163.3[a]	103.6[b]	181.7[c]	161.4[d]	98.6[e]	163.5*	94.1	157.4	103.8	122.5[f]	128.0	121.6[f]	160.6[d]	111.7	130.9
II	164.2[a]	102.5[b]	182.0[c]	160.4[d]	98.9[e]	161.6[d]	103.7[b]	154.3	103.6	121.2[f]	128.0	115.8	161.0[d]	115.8	128.0
Podocarpusflavone															
I	163.9[a]	103.2[b]	181.6[c]	161.5[d]	98.9	163.3[a]	94.0	157.4	104.1	120.1[e]	127.7	121.3[e]	159.4	116.3	131.3
II	164.1[a]	103.2[b]	182.1[c]	160.6[d]	98.9	161.9[d]	103.2[b]	154.6	103.9	123.1	127.9	114.5	162.3[d]	114.5	127.9
Isoginkgetin															
I	163.4[a]	103.7[c]	181.8[e]	161.9[b]	98.8[d]	163.1[a]	94.2	157.5	103.8[c]	122.6	128.2	121.7	160.7[b]	111.5	130.8
II	164.3[a]	103.3	182.1[e]	160.5[b]	99.0[d]	161.7[b]	103.9[c]	154.4	103.8[e]	122.9	127.8	114.4	162.3[b]	114.5	127.7
Ginkgetin															
I	163.5[a]	103.5[e]	181.9[c]	161.5[b]	98.5[d]	165.1	92.6	157.3	104.7	122.3[f]	128.2	121.7[f]	160.6[b]	111.7	130.7
II	163.6*	102.5	182.0[c]	160.4[b]	98.6[d]	161.7[b]	103.8[e]	154.3	103.5	121.2[f]	128.0	115.8	161.0[b]	115.8	128.0
Tetramethyl Amentoflavone															
I	162.5[a]	103.1[d]	181.9[c]	161.4[b]	98.0	165.1	92.7	(157)	104.7[d]	122.4[e]	128.2	121.2[e]	161.1[b]	111.7	130.8
II	163.4[a]	103.1[d]	182.2[c]	160.4[b]	95.5	161.1[b]	103.9[d]	153.5	104.0[d]	122.6	127.8	114.5	162.2[b]	114.5	127.8
Robustaflovone															
I	164.3[c]	103.2	182.1[a]	161.8[b]	99.2	164.0[c]	94.4[d]	157.7	104.1	121.2[e]	127.6	121.3[e]	159.5	116.5[f]	131.2
II	164.5[c]	103.2	182.2[a]	160.0[b]	109.3	162.5[c]	93.6[d]	156.8	103.9	121.6[e]	128.6	116.4	161.5[b]	116.4[f]	128.6
Naringenin*	78.4	42.0	196.2	163.6	95.9	166.7	95.0	162.9	101.8	128.9	128.2	115.2	157.8	115.2	128.2
2,3-Dihydro amentoflavone															
I	78.8	42.5	196.5	163.6[b]	96.0	166.8	95.2	163.2[b]	101.9	128.7	127.8	119.2	156.2	115.9	131.6
II	163.7[b]	102.6	182.2	160.4	98.8	162.2	104.9[a]	154.5	103.7[a]	121.5	128.4	115.9	161.2	115.9	128.4

** Spectra of reference standard.*

[a,b,c,d,e,f] Assignments bearing the same superscript in anyone may be reversed.

substituent effects for the interflavonoid linkage: (1) amentoflavones, I-3′ +6 ppm, II-8″ +10 ppm; (2) robustoflavone, I-3′ +5 ppm, II-6″ +10 ppm; (3) dihydroamentoflavone, I-3′ +4 ppm, II-8″ +9 ppm. Substituent effects which appear to be the best indicators of specific O-methylation in amentoflavone include: (1) I-4′-O-methylation, I-5′ −4 ppm, I-1′ +2 ppm; (2) II-4‴-O-methylation, II-3‴, 5‴ −1.5 ppm, II-1‴′ +2 ppm; (3) 1-7-O-methylation, I-8 −1.5 ppm; (4) II-6″ −3.5 ppm. All the results have been summed up in Table 2.5.

2.4 CONCLUSION

In conclusion, a number of separation, purification, and identification techniques that have been applied to biflavanoids have been reviewed completely. However, in order to carry out full profiling of the minor constituents and further exploration of their natural resources, there is a need for robust and powerful techniques or hyphenated techniques like LC−MS/MS and LC−NMR.

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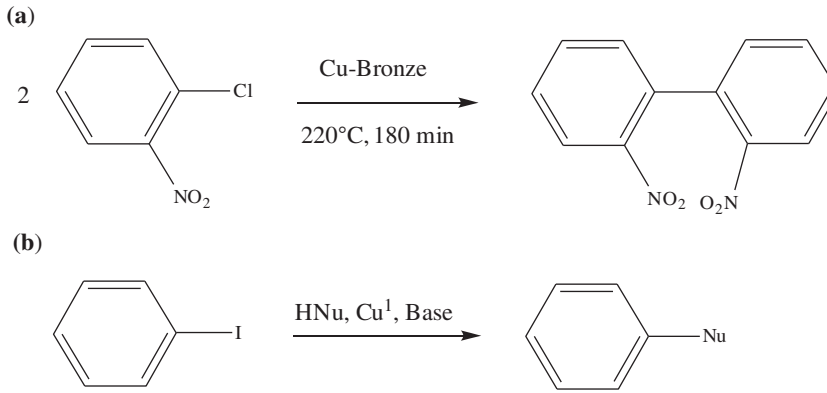
CHAPTER 3

Synthesis of Biflavanoids

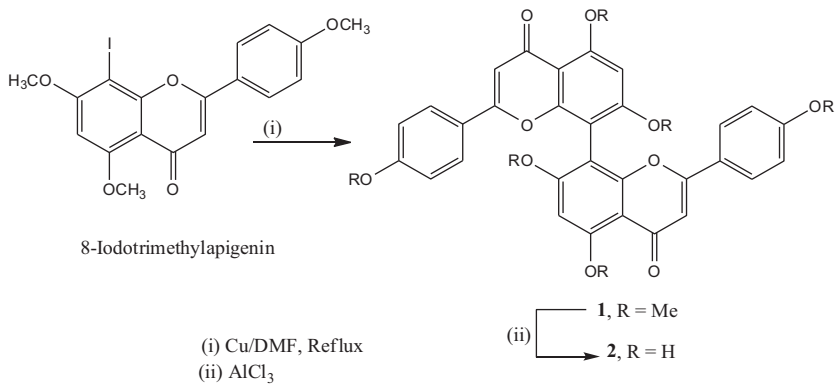
3.1 ULLMANN COUPLING OF HALOGENATED FLAVONES

Ullmann coupling, involves a reaction of aryl halide mediated by elemental copper. A typical example of the Ullmann reaction is the coupling of 1-chloro-2-nitrobenzene with Cu-bronze alloy to yield 2,2′-dinitro-1,1′-biphenyl (Scheme 3.1). This reaction has been classified into two major categories: the "Classic coupling" reaction of aryl halides to give symmetrical biphenyls (Scheme 3.1) and the "modified" reaction which involves Cu-catalyzed coupling of aryl halide and a nucleophile (Scheme 3.1).

The classical Ullmann reaction is of particular interest in the synthesis of biflavanoids, with its major drawback being the requirement of high temperature in the region of 260−300°C, resulting in considerable resinification and poor yields. Nakazawa and Ito investigated the effect of various solvents on the coupling of monoflavones, and found that DMF and DMSO worked best, giving yields of 30% [1].



Scheme 3.1 (a) Classical and (b) Modified Ulmann reaction.

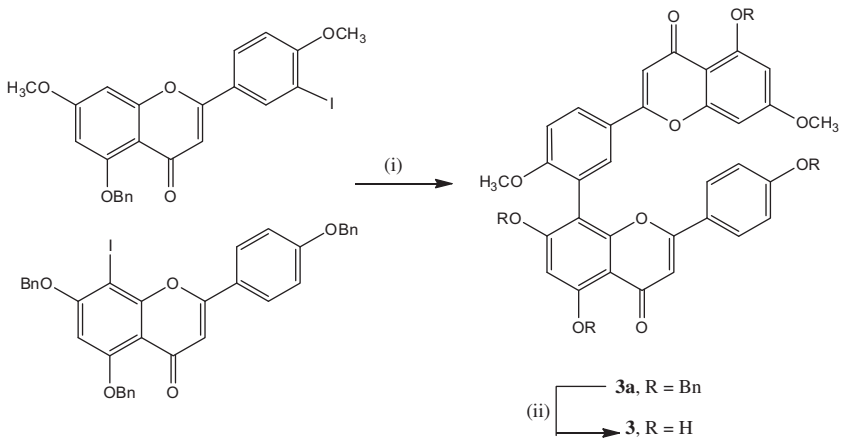


*Scheme 3.2 Synthesis of cupressuflavone hexamethyl ether (**1**) and cupressoflavone (**2**).*

The Ullmann reaction has been used to synthesize symmetrical biflavones. For example, cupressuflavone hexamethyl ether (**1**), a symmetrical biflavanoid, was prepared in 33% yield [2,3]. However, Zhang et al. have carried out the synthesis of cupressoflavone (**2**) improving the yields [4] (Scheme 3.2).

Generally, the bromoflavones and iodoflavones have been found to yield biflavones under the Ullmann conditions. The chloroflavones being unreactive because of the higher electronegativity of the Cl substituent, makes the formation of bonds more difficult. Although the coupling of iodoflavones is expected to be higher-yielding than that of the bromoflavones, this is not observed because of a slightly higher level of reductive dehalogenation.

The Ullmann reaction has also been studied for the synthesis of the unsymmetrical biflavone ginkgetin (**3**). Condensation of the two iodinated flavones using activated Cu powder in DMF unexpectedly resulted in only the symmetrical coupled product. However, omission of the solvent completely changed the course of the reaction and the ginkgetin (**3**) was obtained in 21% overall yield [1] (Scheme 3.3). Following the initial success, a number of unsymmetrical biflavanoids were synthesized [5,6]. However, the overall yields remained low because of the competing symmetrical coupling, as well as due to resinification that accompanies the high temperature conditions.



(i) Cu, neat, reflux, (ii)10% H_3PO_4, AcOH, 110°C

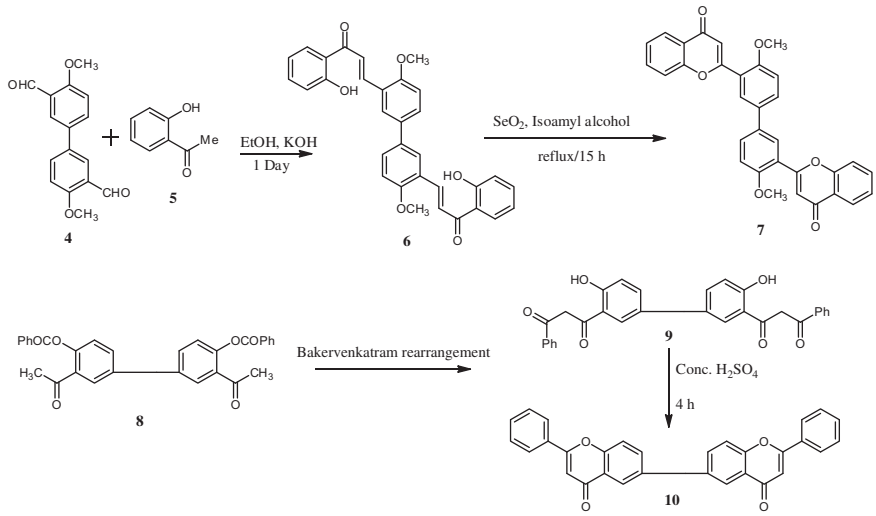
Scheme 3.3 Synthesis of Ginkgetin by Ullmann coupling.

3.2 SYNTHESIS OF BIFLAVONES VIA 1,1′-BIPHENYLS

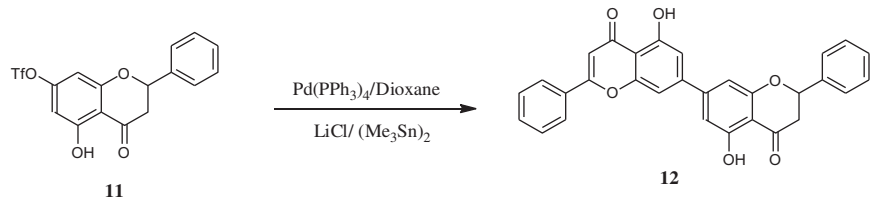
The approach of utilizing a 1,1′-biphenyl skeleton to the synthesis of biflavones was first carried out by Mathai et al. [7,8]. 4,4′-Dimethoxy-3,3′-diformyl-1,1′-biphenyl (**4**) was condensed with 2-hydroxyacetophenone (**5**) in the presence of ethanolic KOH to give the bichalconyl derivative (**6**), which, on refluxing with SeO_2 in amyl alcohol gave the symmetrical 3′,3′-biflavonyl derivative **7** [7] (Scheme 3.4). Several scientists have followed the Mathai approach to synthesize a number of biflavanoids with a variety of modifications, requiring less reaction time, higher yields, and easier separations [9–16].

3.3 METAL-CATALYZED CROSS-COUPLING OF FLAVONES

Stille and Suzuki reactions have been utilized to synthesize the biflavones involving cross-coupling of aryl groups. Biflavone **12** has been synthesized by Stille using cross-coupling of flavone triflate **11** with 0.5 equiv. of distannane [17] (Scheme 3.5). Flavone triflates have been found to cross-couple with a variety of organostannanes under neutral conditions in the presence of LiCl and a Pd^0 catalyst. The technique tolerates various functional groups including alcohol, ester, nitro, acetal, ketone, and aldehyde. The concentration of hexamethylditin



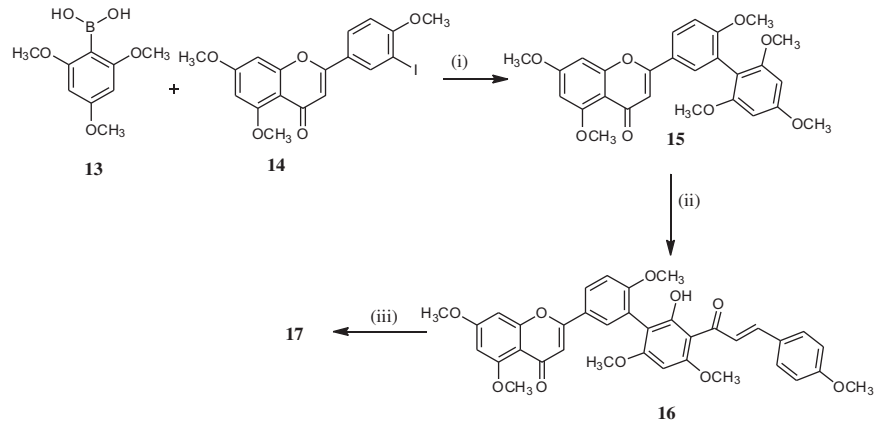
Scheme 3.4 Synthesis of biflavones prepared via biphenyl heteroannulation procedure.



Scheme 3.5 Synthesis of biflavanoids using the Stille reaction.

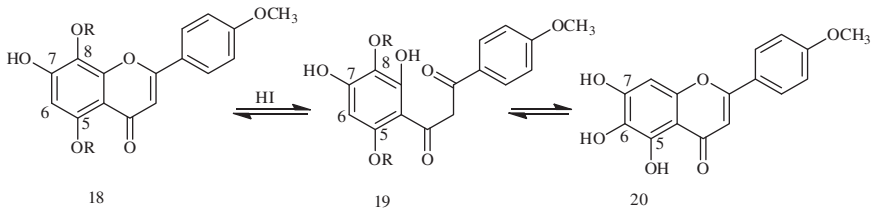
determines whether an aryl(trimethyl)stannane or a symmetrical biaryl is formed. Echavarren and Stille standardized all Pd-catalyzed cross-coupling reactions with organostannanes into two methods involving the use of $Pd(PPh_3)_4$ or $PdCl_2(PPh_3)_2$ in either dioxane or DMF, respectively [17].

Suzuki coupling, which utilizes arylboronic acids, has also been used to synthesize biflavanoids [18] (Scheme 3.6). For example, the boronic acid **13** was coupled with iodoflavone **14**, followed by Lewis acid-catalyzed acylation of **15** with *p*-methoxycinnamic acid. In the course of acylation, regioselective demethylation of the di*ortho*-substituted MeO group occurred to give chalcone **16**, from which the second flavone ring of biflavone **17** was built through a standard oxidative cyclization reaction [18] (Scheme 3.6).



(i) K_2CO_3, $Pd(TPP)_4$, (ii) 4-MeO-C_6H_6-CH=CH-COOH, BF_3Et_2O, (iii) DMSO, H_2SO_4

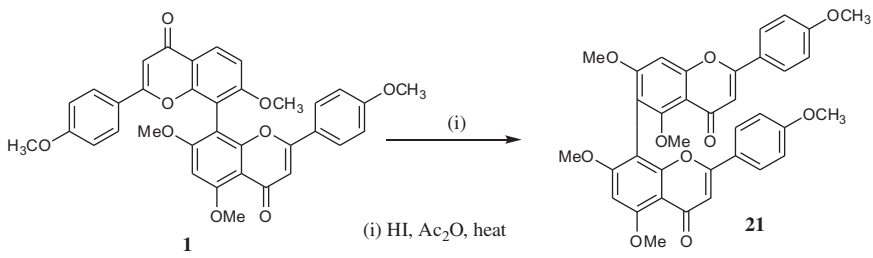
Scheme 3.6 Synthesis of bioflavonoids using Suzuki coupling.



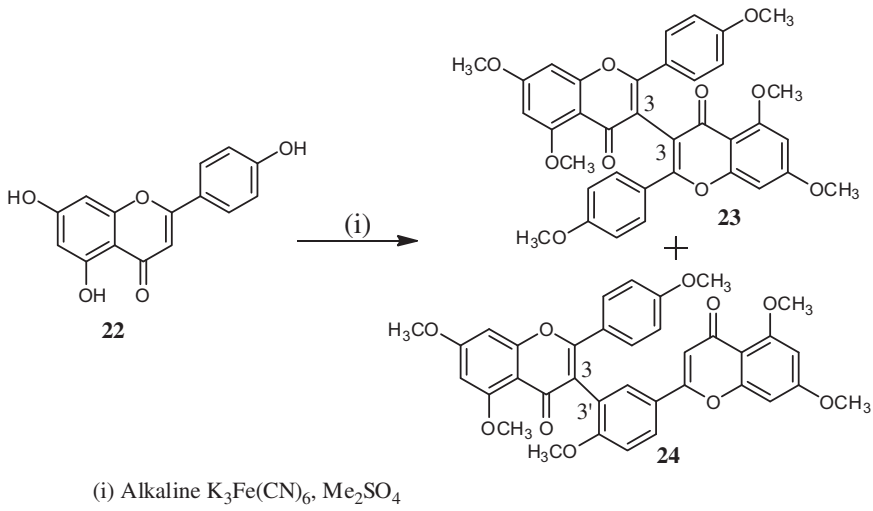
Scheme 3.7 Wessely–Moser rearrangement.

3.4 WESSELY–MOSER REARRANGEMENTS

The Wessely–Moser rearrangement (Scheme 3.7) of monoflavonoids involves the rearrangement of 5,7,8-substituents to a 5,6,7-substitution pattern under acidic conditions. In this rearrangement, the heterocyclic ring of the monoflavanoid undergoes acid-catalyzed ring opening, followed by recyclization with either of the two *ortho*-OH groups to give an equilibrium mixture of the two substitution patterns. Nakazawa has shown that HI in Ac_2O can convert the C(4′)–O–C(8) isomer of hinokiflavone pentamethyl ether into the natural C(4′)–O–C(6) hinokiflavone via the Wessely–Moser rearrangement [19]. Pelter et al. treated (+)-cupressuflavone hexamethyl ether **1** with HI at 130–140°C for 8 h, followed by permethylation to give a mixture of (±)-agithisflavone hexamethyl ether **21** and (±)-cupressuflavone hexamethyl ether **1** in a ratio of 3:2 (*w*/*w*) [20] (Scheme 3.8).



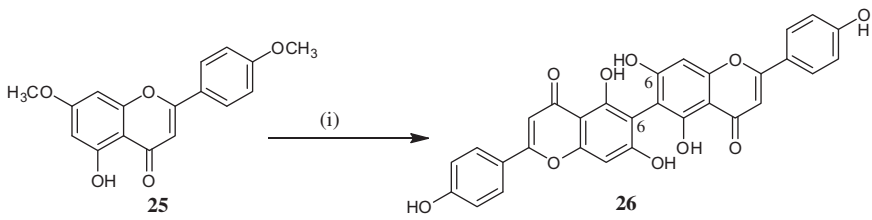
Scheme 3.8 Wesler–Moser rearrangement of (±)-cupressuflavone hexamethyl ether.



Scheme 3.9 Synthesis of biflavanoids using phenol oxidative coupling.

3.5 PHENOL OXIDATIVE COUPLING OF FLAVONES

Phenol oxidative coupling of flavones offers the most pleasing and aesthetic route to the biflavones since it most closely follows the process which is believed to occur in nature. Secondly, this process works on an unprotected polyphenol skeleton unlike several other synthetic approaches which involve the protection–deprotection strategies. It actually involves oxidative coupling of phenolic monomers using one electron oxidizing agents. For example, phenol oxidative coupling of Apigenin (**22**) using alkaline potassium ferricyanide yielded biflavones **23** and **24** [21] (Scheme 3.9). The authors have put forth that this interflavone linkage has occurred through coupling of two radicals. However, such interflavone linkages have not been reported so far in



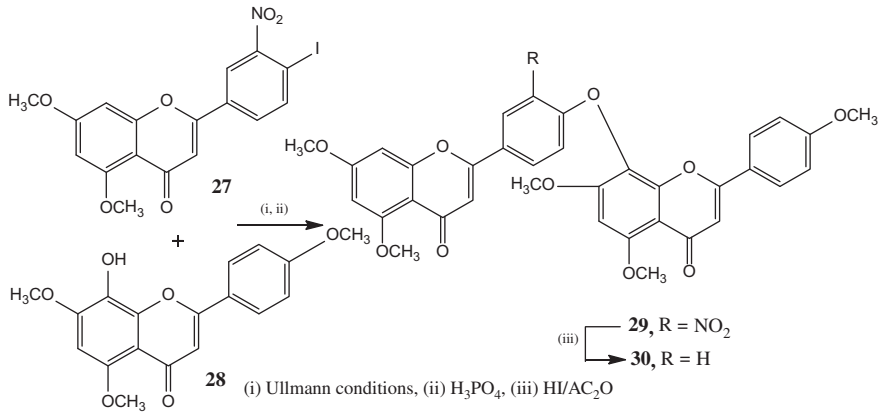
(i) $FeCl_3$ in boiling dioxane

Scheme 3.10 Oxidative dimerization of apigenin-4′,7-dimethyl ether with $FeCl_3$ in dioxane.

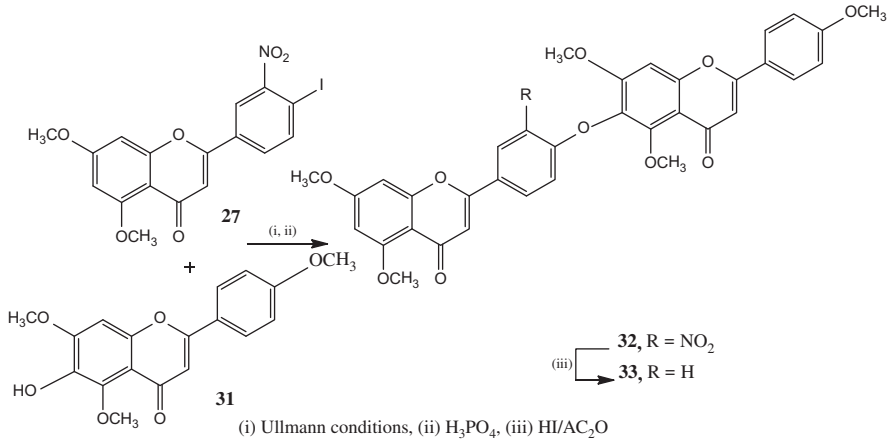
nature. ESR studies have shown that delocalization of an unpaired electron at the C(4′)−OH group in apigenin (**22**) occurs only in the B and C ring [21]. Therefore, the radical generated on the C(4′)−OH group of apigenin (**22**) can delocalize to C(3′), C(1′), or C(3) via oxidative coupling. This radical then attacks the electron rich C(6)− and C(8)− atoms of the A ring which results in the electrophilic substitution, rather than electron pairing. Other researchers have suggested that radical generation on the A ring of apigenin (**22**) is possible when OH groups at C(4′) and C(7) are protected with Me groups. This is justified by the oxidative dimerization of apigenin-4′,7-dimethyl ether **25** with $FeCl_3$ in boiling dioxane that gives a coupled biflavone **26** in 6% yield [2] (Scheme 3.10).

3.6 ULLMANN CONDENSATION WITH FLAVONE SALTS

This modified Ullmann reaction involves the Cu-catalyzed coupling of a halogenated flavone with a nucleophilic flavone salt. This process thus provides a heteroatom-linked biflavanoid in a single step. Introduction of an electron-withdrawing NO_2 group ortho to the halogen atom greatly enhances the reactivity of the halogen atom for Ullmann condensation. For example, flavones **27** and **28** have been coupled under the modified Ullmann conditions to give **29** in 85% yield using DMSO as the high boiling solvent. Compound **29** when subjected to **r**eductive elimination of the NO_2 group gave the biflavanoid **30** (Scheme 3.11) [19]. Similarly, the biflavanoid, hinkoflavone pentamethyl ether **33**, was also accomplished by this methodology from the flavones **27** and **31** (Scheme 3.12). This strategy of biflavanoid synthesis has proved to serve as a synthetic strategy of so many complex bioflavonoids [22,23].



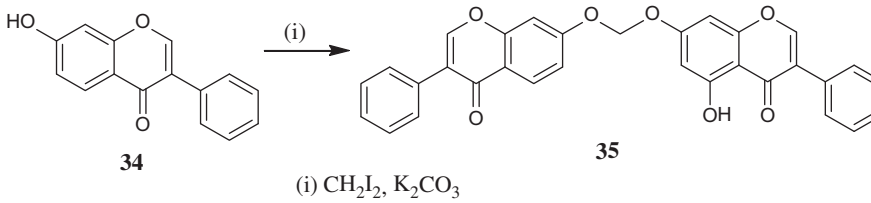
Scheme 3.11 Synthesis of biflavanoid by Ullmann coupling of flavone salts.



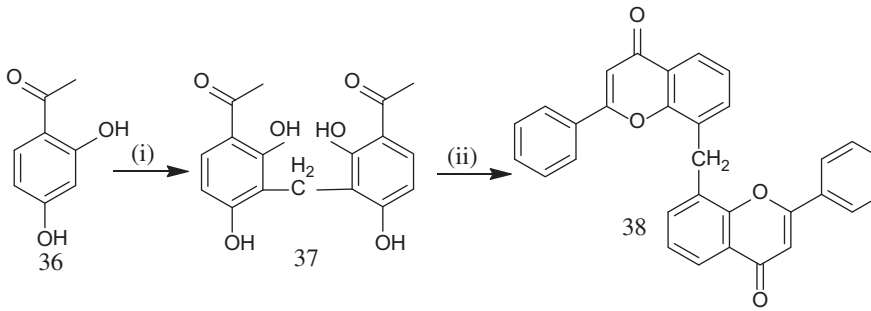
*Scheme 3.12 Synthesis of hinkoflavone pentamethyl ether **(33)** by Ullmann coupling of flavone salts.*

3.7 NUCLEOPHILIC SUBSTITUTION

Methylenation of a phenolic group is a common natural process, often encountered in lignan, alkaloid, and flavanoid biosynthesis. Monomethylenation is straightforward when only two symmetrical OH groups are to be alkylated; however, when the structure contains several OH groups, numerous side reactions make the reaction process difficult [24]. These reactions have been most commonly used to synthesize dialkyl-linked biflavones and biflavanones. The process involves refluxing of monohydroxy-flavones in acetone solution with



*Scheme 3.13 Synthesis of biisoflavone **35** using nucleophillic substitution reaction.*



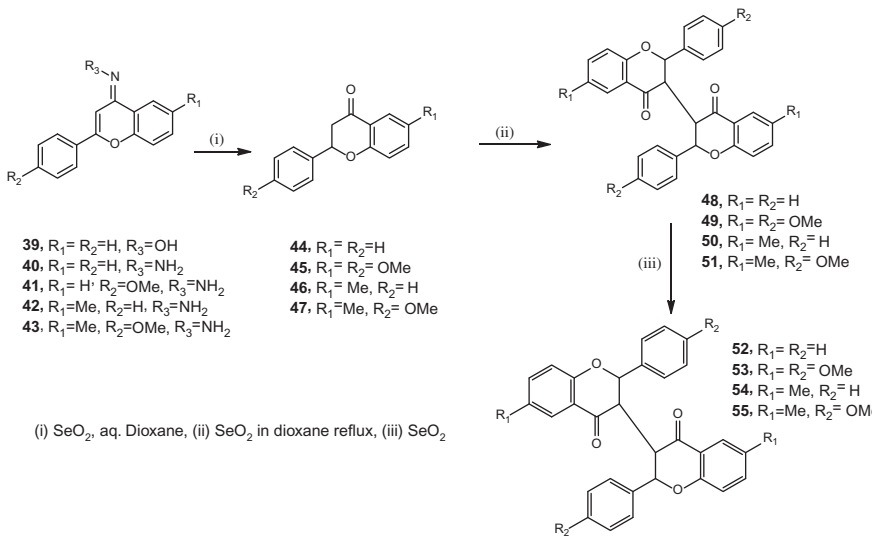
*Scheme 3.14 Synthesis of bisflavonylmethane **38** using nucleophillic substitution.*

alkyl diiodide/dibromide in the presence of K_2CO_3 to produce biflavonyloxyalkanes. Various symmetrical bis(flavonyloxy)-methanes [25–29] and symmetrical biflavanyloxymethanes [27] have been synthesized by this process. An example of these reactions is the synthesis of biisoflavone **35** from its isoflavone **34** as shown in Scheme 3.13.

Similarly Seshadri and coworkers have synthesized a diflavonylmethane **38** (Scheme 3.14) using a nucleophilic substitution reaction. Reaction of resacetophenone (**36**) with CH_2I_2 under alkaline conditions gave the C-methylenated product **37**, which was converted to biflavanoid **38** [24].

3.8 DEHYDROGENATION OF BIFLAVANONES INTO BIFLAVONES

The process of dehydrogenation of the saturated biflavanoid nucleus to the α,β-unsaturated system is an attractive way to synthesize analogs for SAR studies and has also been used to characterize natural biflavanoids. Dehydrogenation is typically achieved with either



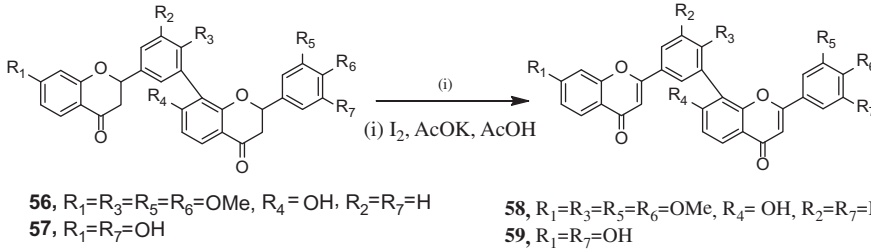
Scheme 3.15 Synthesis of biflavanones and biflavones using the dehydrogenation method.

Fenton's reagent, alkaline potassium ferricyanide, SeO_2, or *N*-bromosuccinimide (NBS). A typical process in this involves the synthesis of biflavone **52**. Oxidation of 4-(hydroxyimino)flavan **39** with SeO_2 in aqueous dioxane gave flavane **44**, which, on oxidative dimerization, resulted in the formation of the biflavone **48**. Finally dehydrogenation with NBS gave biflavone **52** (Scheme 3.15).

Likewise, flavanone hydrazones have also been oxidized with SeO_2 in aqueous dioxane to produce flavones, which, on oxidative coupling and dehydrogenation using NBS, biflavones **52** and **53**–**55** [30] (Scheme 3.15). Alternatively, dehydrogenation of semicarpetin (**56**) and galluflavanone (**57**) was achieved with I_2 and AcOK in AcOH under reflux to generate **58** [31], and **59** (Scheme 3.16) [32].

3.9 HYDROGENATION OF BIFLAVONE INTO BIFLAVANONE

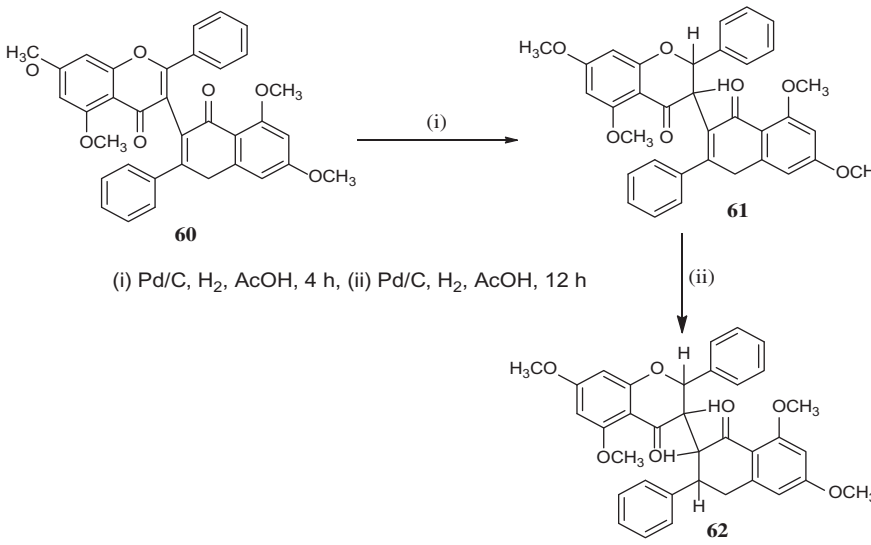
Hydrogenation, which is the reverse of dehydrogenation is also a useful strategy for rapid generation of analogs. However, hydrogenation can be controlled to generate monohydrogenated and dihydrogenated biflavanoids, thereby affording greater structural diversity. For example, catalytic hydrogenation of biflavone **60** when performed at 80°C in glacial AcOH for 4 h (Scheme 3.17) yields monohydrogenated product **62** obtained almost exclusively, however prolonging the reaction for 12 h



56, R_1=R_3=R_5=R_6=OMe, R_4= OH, R_2=R_7=H
57, R_1=R_7=OH

58, R_1=R_3=R_5=R_6=OMe, R_4= OH, R_2=R_7=H
59, R_1=R_7=OH

Scheme 3.16 Synthesis of biflavones from biflavanones using dehydrogenation reaction.



60 **61** **62**

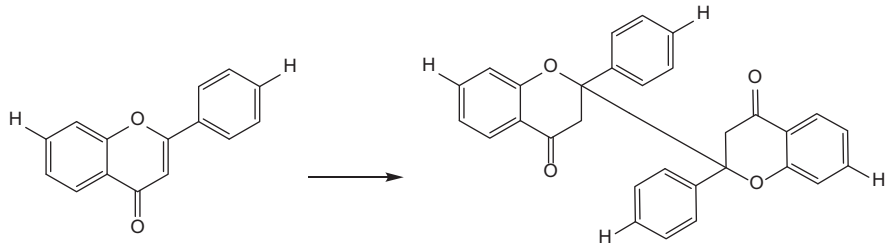
Scheme 3.17 Synthesis of biflavanones from biflavones using the dehydrogenation reaction.

led to both **62** and **61** (Scheme 3.17). However, the yield of the reaction was just 37%. The reason that hydrogenation of **60** is difficult is because the double bond is fully substituted. Li and coworkers have experimented with several hydrogenating conditions including Pd/C−EtOH, Pd/CAcOEt, PtO_2/EtOH, PtO_2/AcOEt, Raney-Ni, $TiCl_3$, and Pd/C−NH_4COO/MeOH, but none of the conditions gave high yields [33].

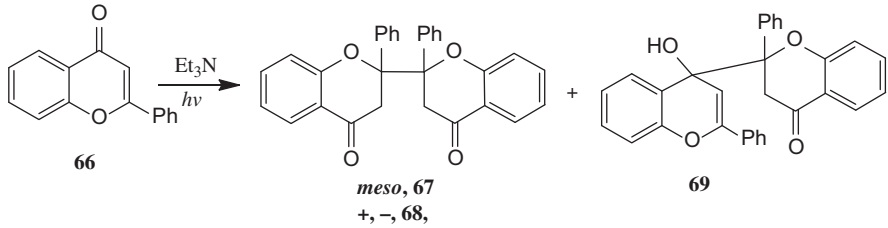
3.10 SPECIAL APPROACHES FOR THE SYNTHESIS OF SELECTIVE BIFLAVANOIDS

3.10.1 Photoreductive Dimerization of Flavones

2,2″-Biflavanones have been synthesized by photoreductive dimerization of flavones. Reductive dimerization of flavone (**63**) in absolute



*Scheme 3.18 Photoreductive dimerization of flavone (**63**).*



Scheme 3.19 Photoinduced electron transfer reactions of flavones with amines.

THF at room temperature under an atmosphere of Argon with *n*-butyl magnesium bromide in the presence of manganous chloride has resulted in a mixture of products (**64** and **65**) in 28% and 38% yields, respectively. The dimerized products are more soluble in dichloromethane than in benzene, ethyl acetate, acetone, ethanol, etc. [34] (Scheme 3.18).

3.10.2 Synthesis of 2,4′- and 2,2′-Biflavanoids Using Photoinduced Electron Transfer Reactions of Flavones With Amines

Photoinduced electron transfer reactions of flavone (**66**) with triethylamine (TEA) in benzene or in acetonitrile has been reported to yield *meso*-2,2′-biflavone (**67**) and (+)-2,2′-biflavone (**68**), as well as 2,4′-biflavanoid (**69**) in good total yield (77% in acetonitrile and 94% in benzene) [35] (Scheme 3.19).

3.11 CONCLUSION

Since the naturally occurring biflavanoids have great biological value, there is a dire need for the rapid synthesis of these molecules.

Although a majority of synthetic procedures are reported, so far these reported strategies focus on preparing only a select group of biflavanoid structures. For example, Ullmann coupling has been exploited to synthesize symmetrical biflavanoid, Stille and Suzuki couplings have been used in the synthesis of their asymmetrical counterparts. Similarly, a nucleophilic substitution approach has been utilized to synthesize diether- or dialkyl-linked flavanoids. However these synthetic procedures suffer from a major drawback of very low yields. Although the available methods have yielded so many diverse biflavanoids, the robust approach that rapidly generates a large number of biflavanoids in higher yields still awaits discovery.

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CHAPTER 4

Biochemical Pharmacology of Biflavanoids

4.1 GENERAL BIOLOGICAL PROPERTIES OF BIFLAVANOIDS

Biflavanoids possess a diverse array of pharmacological activities. They include antibacterial, antifungal, antiallergic, antiviral, antihepatotoxic, anticancer, and immune suppressive activities. For example, amentoflavone has been first reported to strongly inhibit cAMP and cGMP phosphodiesterases with IC_{50} of 0.66 and 0.54 μM, respectively [1]. Biflavanoids isolated from *Ginkgo*, which include ginkgetin amentoflavone, sequoiaflavone, and bilobetin, have been found to inhibit cAMP phosphodiesterase from rat adipose tissue [2]. These have also been very recently found to inhibit cGMP-specific phosphodiesterase-5 (PDE-5) [3]. Amentoflavone is known to inhibit lens aldose reductase [4–6]. Amentoflavone derivatives have been found to be potent inhibitors of human cathepsin B [7], a protease involved in inflammation-related disorders. The biflavanones have been found to be potent tyrosinase inhibitors [8,9]. Amentoflavone strongly antagonizes nicotine-, acetylcholine-, and barium chloride-induced spasmolytic activity of isolated guinea pig ileum [10]. Amentoflavone has been repeatedly found to produce vasorelaxation [11]. The biflavones from *Ginkgo biloba* leaves, including sequoiaflavone, stimulate lipolysis in 3T3-L1 adipocytes [12]. Interestingly, bilobetin and sciadopitysin from *Cephalotaxus koreana* enhance osteoblast differentiation [13], suggesting their therapeutic potential in osteoporosis. Some biflavanoids are known to exhibit antioxidative activity. For example, amentoflavone has been reported to scavenge superoxide radicals [14] and to inhibit nonenzymatic lipid peroxidation [15]. This compound has been found to inhibit CCl_4-induced microsomal lipid peroxidation [16]. Furthermore, sciadopitysin and ginkgetin/isoginkgetin are reported to protect human skin fibroblasts from UV B–induced cytotoxicity, probably by antioxidative mechanism [17].

4.2 ANTICANCER ACTION OF BIOFLAVONOIDS

Biflavanoids are also reported to exhibit potential cytotoxic/anticancer activity. Ginkgetin (**1**) has been found to be cytotoxic to human ovarian adenocarcinoma (OVCAR)-3 cells, but not to other cells like Hep G2 and HeLa [18]. Taiwanhomoflavone-A shows cytotoxicity against several cancer cell lines [19]. The cytotoxic effect of ginkgetin is said to proceed via apoptotic cell death by caspase activation [20]. However, some biflavanoids like sciadopitysin enhance proliferation of normal human skin fibroblasts and increase collagen production [21]. All these results indicate that certain bioflavonoids more profoundly affect cancer cells/cancer cell lines with reduced effects on normal cell proliferation, suggesting their therapeutic potential against cancer.

4.3 ANTIMICROBIAL AND ANTIVIRAL ACTIVITY OF BIOFLAVONOIDS

Some naturally occurring and synthetic biflavanoids show antibacterial, antifungal, and antiviral activities. Several biflavones have antituberculosis activity [22]. The biflavanone, 7,7″-di-*O*-methyltetrahydroamentoflavone, is reported to exhibit weak antimalarial activity [23]. 7,4′-Dimethylamentoflavone (putraflavone) has been found to inhibit *Leishmania mexicana* promastigotes [24]. Furthermore, lanaroflavone possesses antimalarial and leishmanicidal activities [25]. Several ethylene glycol-ligated flavanoid dimers based on the apigenin moiety inhibit *Leishmania* [26]. Conjugates of ginkgetin and sialic acid have been found to possess significant antiviral activity [27]. Very recently, ochnaflavone 7″-*O*-methyl ether and 2″,3″-dihydroochnaflavone 7″-*O*-methyl ether have been found to inhibit HIV-1 activity as well as HIV-1 reverse transcriptase activity [28]. One invention by Flavin et al. [29] reports the antiviral activity of purified antiviral biflavanoids robustaflavone, hinokiflavone, amentoflavone, agathisflavone, volkensiflavone, morelloflavone, rhusflavanone, and succedaneaflavanone. The invention features the antiviral activity of these naturally occuring biflavanoids as well as their derivatives, particularly the salts derived from these biflavanoids. It was found in this invention that these compounds can be used for treating and/or preventing a broad range of viral infections, such as Influenza A and B, hepatitis B, HIV-1, HSV-1, HSV-2, VZV, and measles. It has been discovered that robustaflavone effectively inhibits activity of influenza A and B viruses, hepatitis B, HIV-1, HSV-1, and HSV-2.

Hinokiflavone and morelloflavone exhibited similar activity against various strains of HIV-1. The results have evidenced that robustaflavone is a more potent and effective anti-HIV agent compared to the standard anti-HIV drug used. It has been observed that robustaflavone exhibited an impressive in vitro activity against extracellular (virion) HBV DNA, with an effective average concentration (EC_{50}) of 0.25 μM and an average selectivity index (CC_{50}/EC_{90}) of 153; compared to an effective average EC_{50} of 1.4 μM and average SI of 31 for the control drug. However, amentoflavone, agathistflavone, hinokiflavone, volkensiflavone, rhusflavanone, and succendaneaflavanone possessed little or no anti-HBV activity.

4.4 ANTIINFLAMMATORY ACTIVITY OF BIOFLAVONOIDS: CELLULAR MECHANISMS OF ANTIINFLAMMATORY BIOFLAVONOIDS

Various bioflavonoids, including amentoflavone, bilobetin, sciadopitysin, and ginkgetin, inhibit mast cell histamine release in the micromolar range [30], suggestive of their antiallergic action. Podoverine B, a flavanone–flavonol dimer isolated from plant callus culture, inhibits macrophage chemiluminescence with an IC_{50} of 6.4 μM [31]. Some biflavanoids inhibit lymphocyte proliferation in vitro at 10–100 μM [32]. The biflavanoids including ochnaflavone, ginkgetin, and isoginkgetin inhibit both T-cell and B-cell proliferation induced by mitogenic stimulation without affecting cell viability. The inhibition is irreversible, in contrast to the completely reversible inhibition of T-cell proliferation by flavones/flavonols. These results suggested that certain biflavanoids are general inhibitors of lymphocyte proliferation. Biflavanoids have been found to inhibit mixed lymphocyte reaction. These previous studies indicate that certain biflavanoids are possible therapeutic agents against some allergic and deleterious autoimmune disorders such as rheumatoid arthritis and lupus erythematosus. The effects of biflavanoids on arachidonate metabolic pathways have also been examined since the reaction products, arachidonic acid (AA) and eicosanoids, are pivotal mediators of inflammation. For the first time, ochnaflavone and several other biflavones have been found to inhibit secretory phospholipase A2 (sPLA2-IIA) [33], and some of them actually inhibit AA release from mouse peritoneal macrophages in culture [34]. Morelloflavone, a biflavanoid, has also been found to inhibit sPLA2 and has in vivo antiinflammatory activity in animal models of

12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema and carrageenan (CGN)-induced paw edema in mice [35]. Notably, this compound shows in vivo activity by oral administration. Ginkgetin, a ginkgo biflavanoid, has been found to inhibit cytosolic PLA2 (cPLA2). Ginketin generally inhibits epidermal cPLA2 of guinea pig skin [36]. Cyclooxygenase (COX) produces prostanoids, and lipoxygenase (LOX) synthesizes HETEs and leukotrienes. Many nonsteroidal antiinflammatory drugs and some antiallergic drugs that are used clinically are inhibitors of COX/LOX. When the effects of biflavanoids on COX-1/12, 15-LOX were examined using guinea pig epidermal homogenate as an enzyme source, amentoflavone was found to be a potent COX-1 inhibitor ($IC_{50} = 3\ \mu M$) compared to indomethacin ($IC_{50} = 1\ \mu M$), while ginkgetin only weakly affected COX-1 [37]. Another similar finding of COX-1 inhibition by amentoflavone has also been reported [38]. Tetrahydroamentoflavone has been demonstrated to be a weak COX-1/COX-2 inhibitor [39]. On the other hand, there has been only one report of inhibitory activity by biflavanoids against LOXs. Ginkgetin has been reported to be an effective 5-LOX inhibitor at the cell-level [40]. However, the detailed LOX inhibitory activity of bioflavonoids awaits discovery. All of these facts have demonstrated that certain natural biflavanoids possess antiinflammatory activity via inhibition of eicosanoid metabolizing enzyme activities, thereby reducing concentrations of proinflammatory mediators. One important antiinflammatory mechanism of biflavanoids is transcriptional regulation of proinflammatory molecules. The biflavanoids, bilobetin and ginkgetin, were initially found to suppress inducible nitric oxide synthase (iNOS) and COX-2 expression in LPS-treated RAW 264.7 cells [40]. For further elucidation, their effects on iNOS expression has also been studied. Several biflavones, such as ginkgetin, isoginkgetin, bilobetin, and ochnaflavone, have been found to downregulate iNOS expression in LPS induced RAW 264.7 cells, whereas amentoflavone did not [41]. Moreover, ginkgetin has been shown to inhibit COX-2 induction in LPS-treated RAW cells without affecting COX-1 levels [42]. After these reports, continual findings of regulatory properties of biflavanoids on proinflammatory gene expression have been described, as summarized in Table 4.1. Some of these suppressive properties of bioflavonoids against proinflammatory molecules have been confirmed in vivo. For example, topical treatment with ginkgetin reduced COX-2 induction in TPA-treated mouse skin [43]. Furthermore, topical application of ginkgetin reduced chronic skin

Table 4.1 Transcriptional Regulation of Proinflammatory Molecules by Biflavanoids

Biflavanoids	Target Cells	Stimulant	Target Genes[a]
Amentoflavone	M1	–	ICAM-1
Amentoflavone	A549	TNF-α	PPAR-γ (↑), COX-2, NF-κB
Ginkgetin	Mouse BMMC	Cytokines	COX-2
Ochnaflavone	Raw 264.7	LPS	iNOS, ERK1/2, NF-κB
Ochnaflavone	HASMC	TNF-α	ERK1/2, MMP-9
Isoginkgetin	HT1080	–	TIMP-1 (↑), MMP-9, PI3K/Akt

M1, mouse myeloid leukemia cells; HASMC, human aortic smooth muscle cells; HT1080, human fibrosarcoma cells.
[a]These proinflammatory molecules are downregulated or inhibited, whereas ↑ indicates upregulation.

inflammation provoked by multiple TPA treatments, with concomitant reduction of edematic response [44]. This reduction has been observed by histological comparison. Therefore, it is now clear that certain biflavanoids downregulate expression of proinflammatory molecules such as COX-2 and iNOS in vitro as well as in vivo. Ogundaini et al. [45] reported the antiinflammatory nature of the isolated biflavanoids like diinsininol and diinsinin. Diinsininol and diinsinin exhibited IC_{50} values of 9.20 and 13.14 μM, respectively, in the inhibition of prostaglandin synthesis assay compared with indomethacin, which had an IC_{50} value of 0.56 μM, and IC_{50} values of 49 and 39 μM in the inhibition of platelet activating factor–induced exocytosis, which makes them more potent inhibitors of PAF-induced exocytosis than the known PAF antagonist ginkgolide BN 52021, isolated from the tree *G. biloba* (IC_{50} of 80 μM).

4.5 ANALGESIC ACTIVITY OF BIOFLAVONOIDS

As an antiinflammatory agent, the compound with analgesic activity is favored. The initial in vivo study of amentoflavone has not revealed any neuropharmacological and neuroanalgesic effects by i.p. injection, suggesting a lack of penetration through the blood–brain barrier (BBB) [10]. Amentoflavone is nontoxic at doses of up to 1.5 g/kg i.p. However, in vitro experiments suggested that amentoflavone might be able to penetrate the BBB by passive diffusion [46]. Biflavanoids, such as amentoflavone and isoginkgetin, have shown neuroprotective effects in vitro [47]. Although it is still not clear whether amentoflavone or other biflavanoids penetrate the BBB, their peripheral analgesic activities

have been demonstrated several times. Some bioflavonoids are known to possess peripheral analgesic activity by i.p. injection. It has been shown that amentoflavone and ginkgetin possess potent analgesic activity against writhings by i.p. injection, but not by oral administration [48,49]. Similarly, 3-8″ binaringenin has shown antinociceptive activity by i.p. injection in the writhing test and formalin test [50]. 13-Naringenin-II8-4′-*O*Me-eriodictyol isolated from *Rheedia gardneriana* leaves has been reported to possess analgesic activity by i.p. injection [51]. Thus, it is clear that some biflavanoids possess analgesic activity, which may lead to the development of better antiinflammatory agents.

4.6 ANTIINFLAMMATORY ACTIVITY OF SYNTHETIC FLAVANOIDS

There have only been a few trials to synthesize a biflavanoid library. Various synthetic methods have been proposed for the synthesis of biflavanoids and have all been discussed in the previous chapter. Some of the synthetic biflavanoids are reported to have shown antituberculosis activity [22]. These compounds have been found to inhibit sPLA2-IIA to varying degrees depending on the position of the C−C connection [52]. Continuing research has shown that they differentially inhibit various isoforms of PLA2, including sPLA2 and cPLA2 [52]. These biflavanoids have also been found to possess inhibitory activity against COX-2-mediated PGE2 production in LPS-treated RAW 264.7 cells. And the results have demonstrated that several derivatives such as A−A and A−B ring biflavanoids have higher inhibitory activity against PGE2 production in LPS-treated RAW cells [53]. In particular, in the micromolar concentration range, 6-6″ C−C biflavone potentially inhibited COX-2 without affecting COX-2 expression. This biflavone is reported to have exhibited in vivo antiinflammatory activity against rat CGN-induced paw edema. However, it did not significantly inhibit iNOS mediated NO production. These studies suggest that C−C biflavones without substitution on the molecule do not have the capacity to regulate the transcription of proinflammatory molecules, such as COX-2 and iNOS. They simply behave as PLA2 and COX-2 inhibitors, in contrast to certain natural biflavanoids that have hydroxyl/methoxyl substitutions on the molecule. Some synthesized bichalcone derivatives have been examined for their PGE2 inhibitory activity.

4.7 CONCLUSION

The research on antiinflammatory biflavanoids is in the early stages and is now expanding. Based on initial studies, biflavanoids utilize multiple antiinflammatory mechanisms. They affect inflammatory cells such as mast cells and lymphocytes. They inhibit proinflammatory enzymes such as PLA2 and COX. Recent investigations also demonstrate that they suppress proinflammatory molecule expression. Due to these unique properties, bioflavonoids have potential as antiinflammatory drugs especially for treating chronic inflammatory disorders. Through more intensive studies with modern pharmacological techniques, new types of antiinflammatory agents based on biflavanoid structures may be successfully developed.

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