Spiro-Flavonoids in Nature

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The research collects 65 unique structures, including spiro-biflavonoids, spiro-triflavonoids, spiro-tetraflavonoids, spiro-flavostilbenoids, and scillascillin-type homoisoflavonoids. Scillascillin-type homoisoflavonoids comprise spiro[bicyclo[4.2.0]octane-7,3′-chromane]-1(6),2,4-trien-4′-one, while the other spiro-flavonoids contain either 2*H*,2′*H*-3,3′-spirobi[benzofuran]-2-one or 2′*H*,3*H*-2,3′-spirobi[benzofuran]-3-one in the core of their structures. Spiro-flavonoids have been described in more than 40 species of eight families, including Asparagaceae, Cistaceae, Cupressaceae, Fabaceae, Pentaphylacaceae, Pinaceae, Thymelaeaceae, and Vitaceae. The possible biosynthetic pathways for each group of spiro-flavonoids are summarized in detail. Anti-inflammatory and anticancer activities are the most important biological activities of spiro-flavonoids, both in vitro and in vivo.

spiro-flavonoids | spiro-biflavonoids | spiro-triflavonoids | spiro-tetraflavonoids | spiro-flavostilbenoids | scillascillin-type homoisoflavonoids | biosynthesis | biological activity | isolation

1. Introduction

A spiro compound, or spirane (from Latin spīra, meaning twist or coil), is an organic compound containing two or more rings connected by only one common carbon atom and exhibiting a twisted structure [1][2]. Spiro-containing substances have central or axial chirality and 3D structural properties in relation to their intrinsic rigidity [3]. These scaffolds have been found to possess pharmacological potencies and a wide range of biological activities. Spiro compounds are of increasing interest in medicinal chemistry and contribute to a large number of approved drugs and drug candidates [4].

Flavonoids have a 15-carbon backbone consisting of two benzene rings (rings A and B) connected by a 3-carbon linker (represented as C_6-C_3-C_6 compounds). This linker can form a heterocyclic C (pyran or pyrone) ring. In the absence of this additional system, such flavonoids are called chalcones and are usually precursors in the biosynthesis of various classes of flavonoids. Spiro-flavonoids, on the other hand, are characterized by the presence of one or more spiro-carbons derived from the C-3 carbon of the corresponding flavonoid. This class of compounds is divided into two main groups: monomers and heterooligomers. Monomeric spiro-flavonoids include only scillascillin-type homoisoflavonoids with the structure spiro[bicyclo[4.2.0]octane-7,3′-chromane]-1(6),2,4-trien-4′-one (**Figure 1**A), which are derivatives of 3-benzylchroman-4-one [5]. The name homoisoflavonoids, which is used for compounds with a 3-benzylchroman-4-one backbone, suggests that they are formed biosynthetically in nature similar to isoflavonoids, that is, by a characteristic 2,3-aryl migration step, while they are formed by

modifying the typical C_6-C_3-C_6 backbone of flavonoids by inserting an additional carbon atom. Depending on the number of flavonoid units, oligomeric spiro-flavonoids are further divided into the following subclasses: spiro-biflavonoids (flavanone-flavan-3-ol or flavan-3-ol-chalcone dimers), spiro-triflavonoids (flavanone-flavan-3-ol trimers), spiro-tetraflavonoids (mixed combinations of flavan/flavan-3-ol-chalcone), and atypical spiro-flavostilbenoids (dimers and flavanone-stilbenoid trimers) [6][7]. The presence of the 2*H*,2′*H*-3,3′-spirobi[benzofuran]-2-one (**Figure 1**B) or 2′*H*,3*H*-2,3′-spirobi[benzofuran]-3-one (**Figure 1**C) systems connects all compounds belonging to the class of oligomeric spiro-flavonoids. To date, no spiro-flavonoids have been described that have an iso- or neoflavonoid unit in their structure.

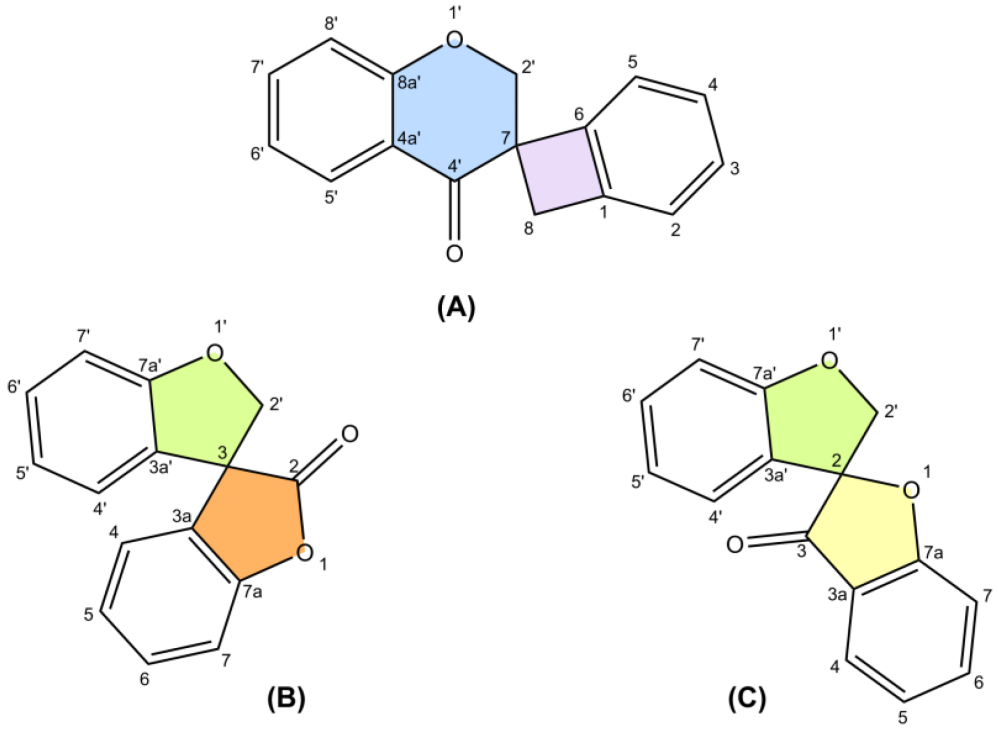


Figure 1. Core structures of monomeric scillascillin-type homoisoflavonoids (**A**) and oligomeric spiro-flavonoids (**B**,**C**).

2. Biosynthesis of Spiro-Flavonoids

Since studies on the biosynthesis of spiro-flavonoids have not yet been performed, it seems appropriate to present possible mechanisms in light of the knowledge of flavonoid biosynthesis. It is generally accepted that flavonoids are synthesized in the cytosol, and the enzymes involved are connected to the endoplasmic reticulum [8][9]. The pathway begins with the formation of the C_6-C_3-C_6 skeleton by chalcone synthase (CHS), which catalyzes the synthesis of naringenin chalcone from one molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA. In the biosynthesis of flavonoids, the unstable chalcone is stereospecifically converted to the flavanone, (2*S*)-naringenin, by chalcone isomerase (CHI). In the absence of CHI, the isomerization of chalcone occurs spontaneously, producing the racemic mixture of (2*S*)-naringenin and (2*R*)-naringenin, but the enzyme-catalyzed reaction is 107 times faster than the spontaneous one [10][11]. (2*S*)-Flavanones are the exclusive substrates of

downstream enzymes of the flavonoid pathway, and thus CHI guarantees the efficient formation of biologically active (2*S*)-flavonoid isomers. Interestingly, *Arabidopsis* mutants lacking CHI activity have been reported to accumulate only trace amounts of flavonoids [12].

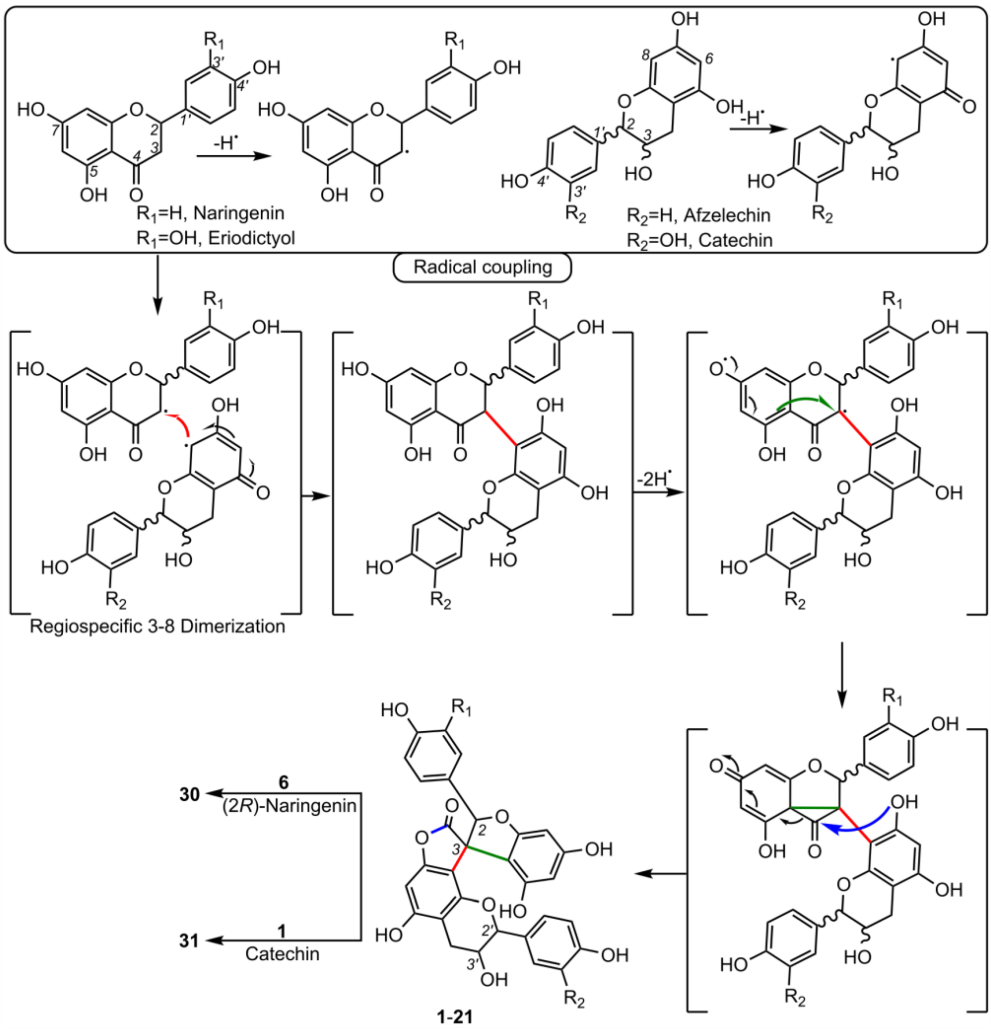
During flavonoid biosythesis, flavanones can undergo desaturation by flavone synthase I, which produces apigenin from (2*S*)-naringenin. Subsequently, flavanones can also be regioselectively and stereoselectively converted to (2*R*,3*R*)-dihydroflavonols (the presence of the 3-hydroxyl group reverses the priority sequence of the Cahn–Ingold–Prelog specification of absolute configuration [13]) via the intermediate C-3 flavanone radical [14] by hydroxylation of a pro-*R* hydrogen in C-3 catalyzed by both flavanone 3β-hydroxylase and flavone synthase [15]. These enzymes are classified as soluble 2-oxoglutarate-dependent dioxygenases due to their requirement for the cofactors 2-oxoglutarate, molecular oxygen, ferric iron (Fe^{2+}), and ascorbate. Only (2*S*)-naringenin, but not the (2*R*)-enantiomer, was reported to be a substrate for flavanone 3β-hydroxylase from *Petunia hybrida* [16]. On the other hand, in addition to the oxidation of *trans*-(2*R*,3*R*)-dihydrokaempferol, but not that of *trans*-(2*S*,3*S*)-dihydrokaempferol to kaempferol, flavonol synthase catalyzes the conversion of (2*S*)- or (2*R*)-naringenin to the corresponding *trans*-dihydrokaempferol [15]. Subsequently, dihydroflavonols are reduced to flavan-3,4-diols by dihydroflavonol 4-reductase and leucoanthocyanidin reductase during the biosynthesis of flavan-3-ols, also involving anthocyanidin reductase and anthocyanidin synthase.

Stilbene synthase is closely related to CHS, catalyzing a similar set of reactions, but performing a divergent cyclization reaction to generate stilbenes with the C_6-C_2-C_6 backbone, which compete with CHS for the same substrates (*p*-coumaroyl-CoA and malonyl-CoA) [17]. There is also evidence that stilbene synthase (STS) has evolved from CHS several times during evolution [9]. Both chalcone and stilbene synthase lead to a tetraketide intermediate in the form of CoA thioester followed by C_6-C_1 Claisen condensation to give chalcone in the case of CHS, while C_2-C_7 aldol condensation with loss of CO_2 leads to resveratrol in the case of STS. Competition between two metabolic pathways, the flavonoid pathway controlled by chalcone synthase on the one hand and the stilbene pathway controlled by STS on the other, may occur with respect to precursor availability. Deprivation of CHS of its substrates results in reduced levels of some flavonoids, such as rutin or naringenin in transgenic tomato, levels of flavonols in recombinant strawberry, or pale fawn color in *Arabidopsis* [18][19][20][21]. The expression of stilbene synthase genes has been shown to contribute to the constitutive defense against pathogens in grapevines [22]. It has been suggested that the formation of resveratrol dimers and higher oligomers, thought to be phytoalexins, is a plant response to stress caused by fungal infection, UV radiation, or physical trauma [23][24][25]. Various modifications can occur on the stilbene core under the catalytic action of peroxidases; this is the basis of the polymerization process leading to the formation of oligomeric structures of resveratrol, as well as other reactions involving “decorating” enzymes (methyltransferases, prenyltransferases, hydroxylases, and glucosyltransferases). This oxidative coupling of resveratrol to form oligomeric compounds was extensively studied in *Vitis vinifera* L. [26] and proceeds through the coupling of oxidatively generated phenoxyl radicals, as originally proposed [27]. The dimerization process can proceed in several regioisomeric coupling modes (3-8′, 8-10′, 8-8′, and 8-12′). After this step, the formed highly reactive *p*-quinone methides can undergo a series of regioselective Friedl–Crafts reactions, nucleophilic trappings, or tatutomerizations, leading to an impressive number of possible

products: resveratrol derivatives. However, the presence of oligomeric forms of resveratrol has not been reported in plants containing spiro-flavonoids that contain a stilbene moiety in the structure.

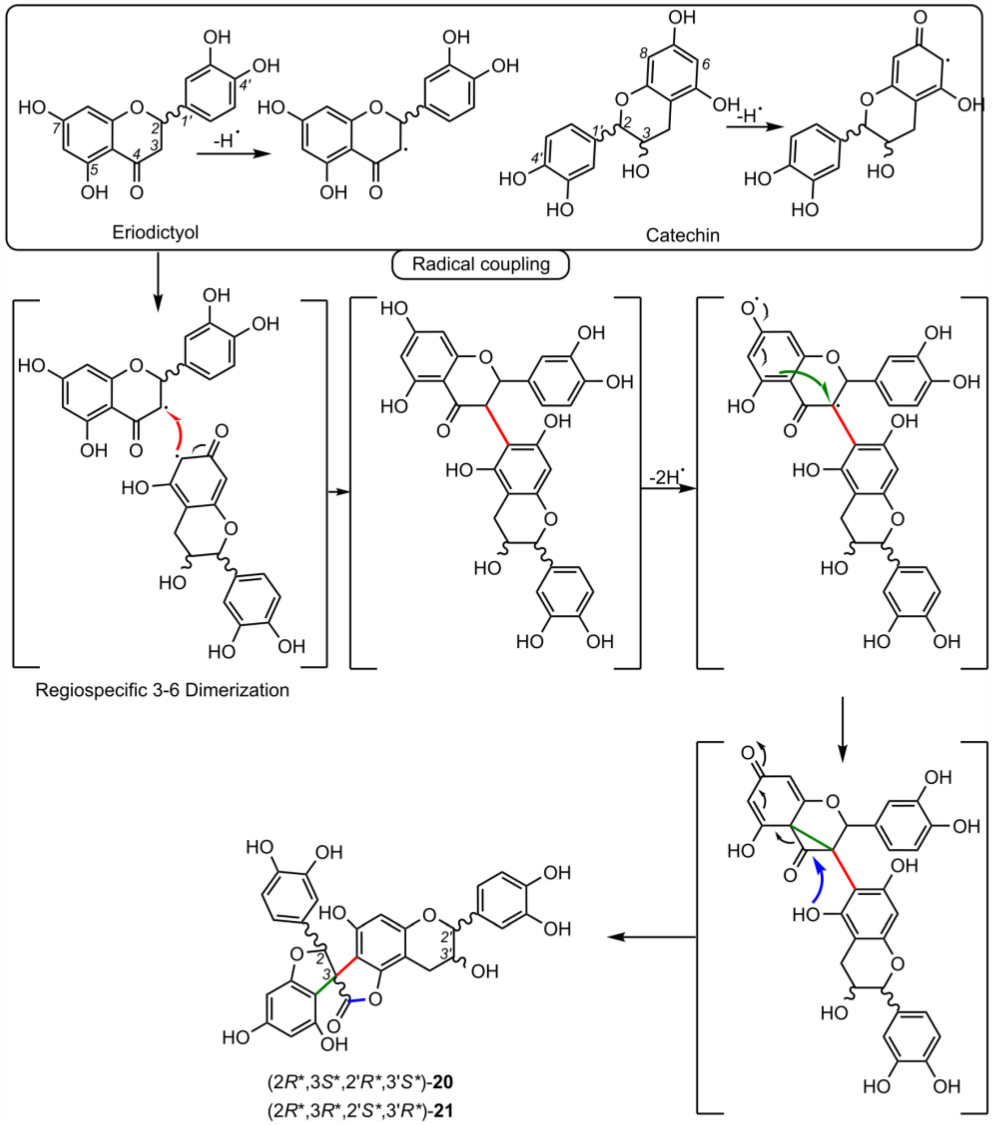
Existing hypotheses regarding the mechanisms of spiro-flavonoid formation require at least two oxidation steps and suggest that these compounds are formed by trapping a putative carbocation intermediate in the oxidative conversion of flavanone to flavonol [28][29], or by radical coupling of two oxidatively generated phenoxyl radicals [7][30]. The hypothesis that assumes the formation of a flat carbocation does not specify how exactly it would be formed. However, the free radical mechanism also produces a flat trigonal radical that can react on either side. In general, the process of formation of spiro-flavonoids may not be directly related to the biosynthesis in the cytoplasm. Their formation outside photosynthetic tissue could be due, as in the case of resveratrol oligomers, to interference with ion transport and related redox processes [31]. Rather, the necessary substrates can be transported across cell membranes to the lignifying zone in the cell wall, where they undergo enzymatic oxidation (dehydrogenation). As in lignin synthesis by radical coupling of monolignols, the enzymes laccase and peroxidase may be involved in this oxidation, but further experimental data should be collected to support these statements [32][33]. Interestingly, Pecio et al. (2023) [7] observed that all spiro-flavonoids isolated from the bark of *Y. schidigera* and the spiro-biflavonoid fragranol B (**8**), as well as the spiro-triflavonoid fragranol A (**30**) from the bark of *Anneslea fragrans*, have (2*R*)-naringenin in their structures [34][35], while abiesinoles A-H from the bark of *Abies sachalinensis* contain (2*S*)-naringenin or (2*S*)-eriodictyol [36]. This indicates a certain stereoselectivity in their formation.

Scheme 1 shows a hypothetical biosynthetic relationship between flavanones, flavan-3-ols, and larixinol-type spiro-bi- and spiro-triflavonoids (**1**–**21**, **30**–**31**, respectively) according to the mechanism proposed by Zhou et al. (2020) and Pecio et al. (2023) [7][30]. Their formation begins with the regiospecific coupling of two radicals (linkage 3–8). In the next step of oxidation, a triplet diradical is formed, which undergoes a Favorskii-type rearrangement by forming a cyclopropanone intermediate [37]. This intermediate is lactonized by nucleophilic substitution to form a benzofuranone ring through preferred *exo*-trig cyclization [38][39].



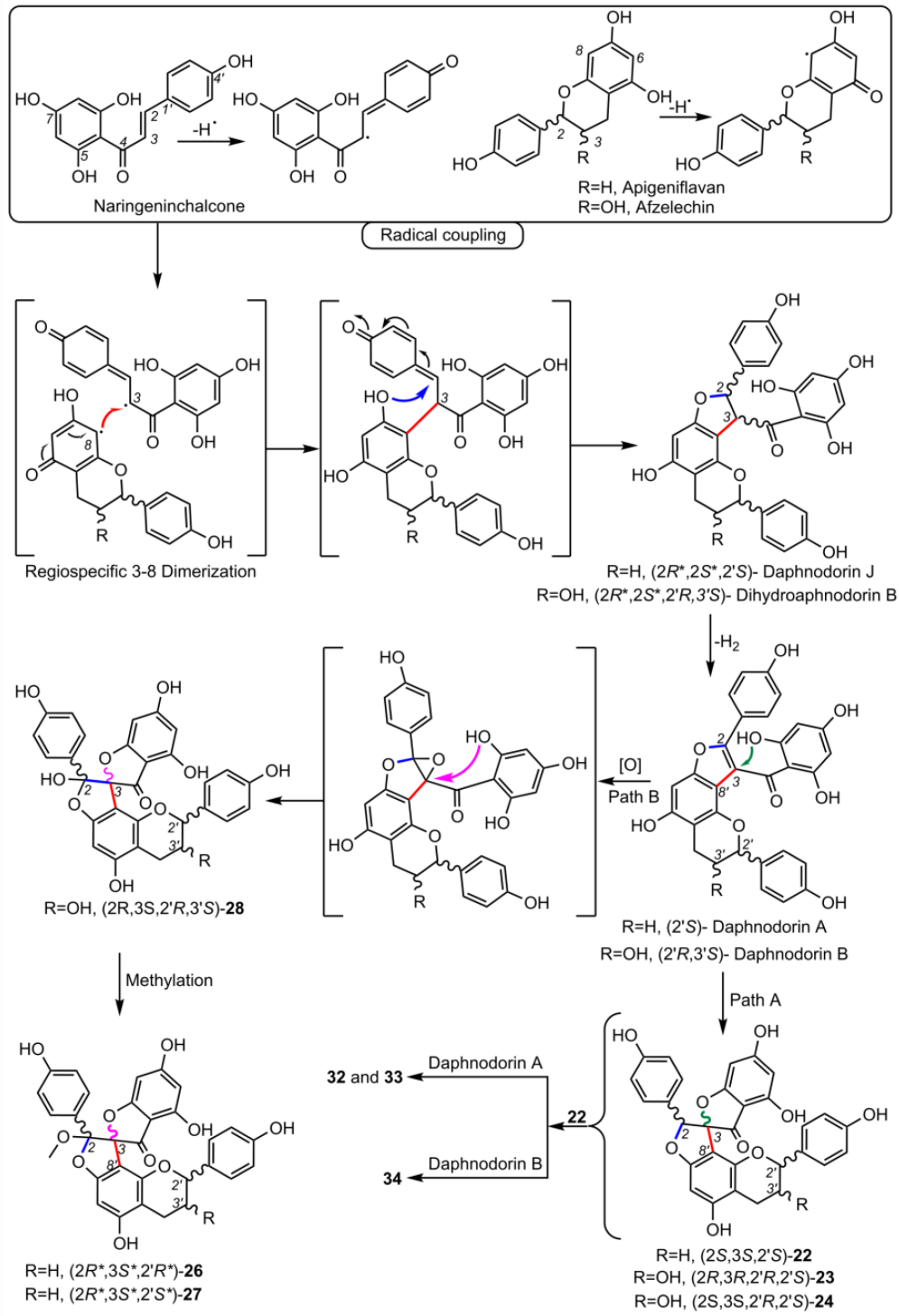
Scheme 1. Hypothetical biosynthetic routes to larixinol-type spiro-flavonoids.

Olgensisinols C and D (**20**–**21**) can be formed similarly, the only difference being that the initial radical coupling requires a 3–6 linkage (Scheme 2).



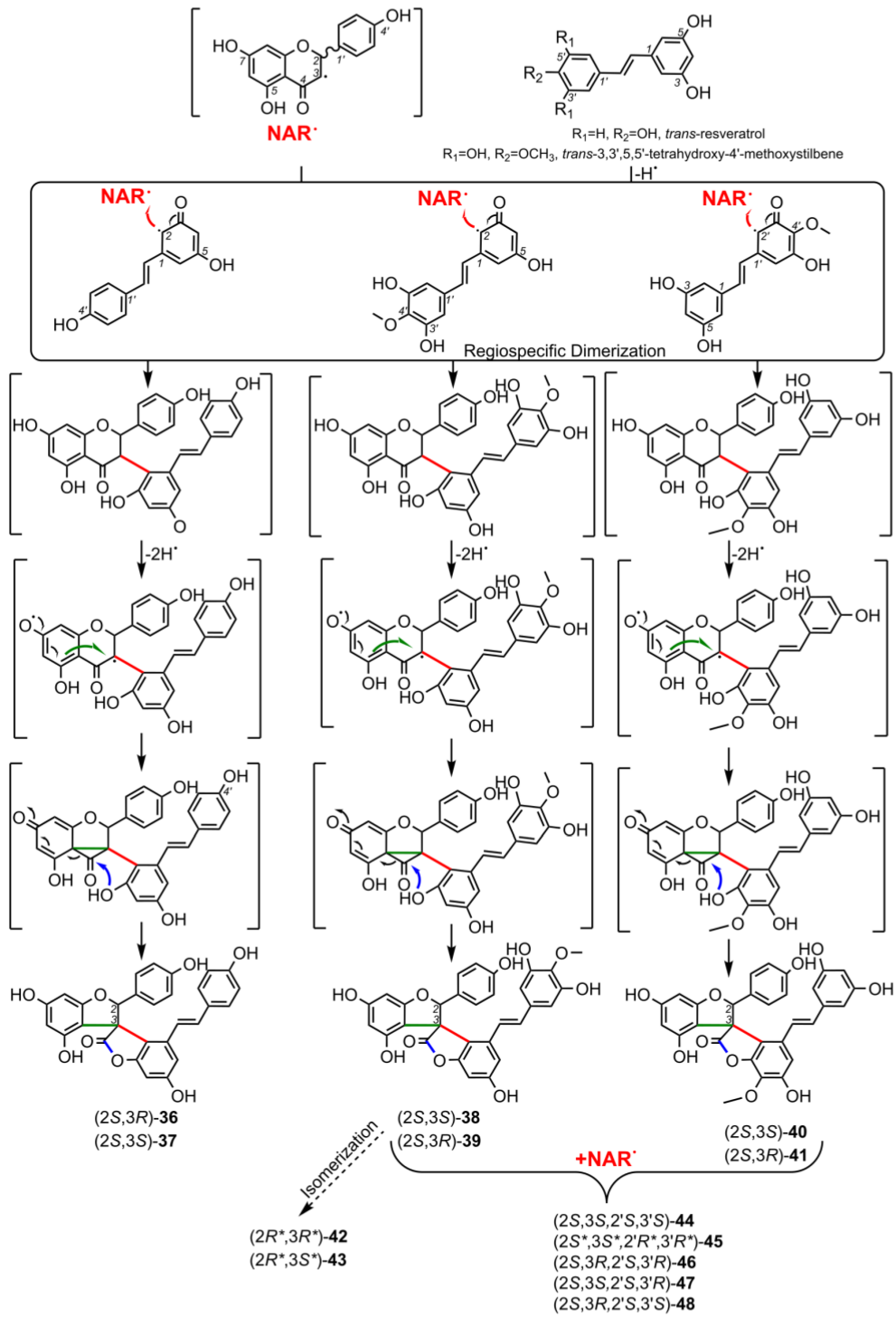
Scheme 2. Hypothetical biosynthetic routes to olgensisinols C and D.

The biosynthesis of the spiro-biflavonoids of the daphnodorin C type (Scheme 3), on the other hand, appears to proceed in a slightly different manner and is initiated by the coupling of the naringeninchalcone radical with the apigeniflavan or afzelechin radical (flavan-3-ol). This results in the formation of an activated *p*-methide quinone, which then undergoes nucleophilic substitution with a hydroxyl group to give a benzofuran ring and two known compounds, daphnodorins B and J. These in turn can be dehydrogenated to the next known compounds, daphnodorins A and B, which undergo two divergent reactions (paths A and B). Path A involves cyclization through the attack of the hydroxy group on the C-3 of the double bond, producing compounds **22**–**24**, while path B involves epoxidation of the double bond C-2/C-3 and subsequent opening of the oxirane ring and simultaneous cyclization to a spiro-type system (**28**).



Scheme 3. Hypothetical biosynthetic routes to daphnodorin C-type spiro-flavonoids.

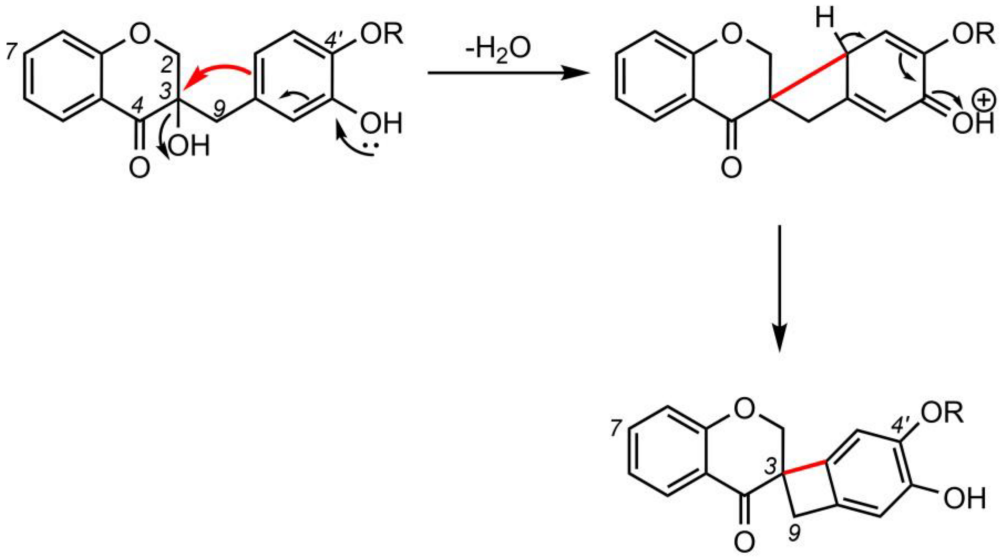
It also appears that the mechanism behind the biosynthesis of the spiro-flavostilbenoids is similar to the one shown in Scheme 1 and Scheme 2. That is, diastereoisomeric pairs of compounds **36**–**37**, **38**–**39**, and **40**–**41** are formed as a result of a regiospecific coupling of the naringenin radical with a stilbene-type radical (resveratrol or THMS). Interestingly, compounds **38**–**41** undergo further coupling with the naringenin radical to give compounds **44**–**48** and probably also form more complex polymeric structures [40] (Scheme 4).



Scheme 4. Hypothetical biosynthetic routes to spiro-flavostilbenoids.

The hypothetical mechanisms of homoisoflavonoids formation are described in detail in the work of Castelli & Lopez [41], to which the reader is referred. However, the only works devoted to the study of the biosynthesis of these compounds (specifically, 3-benzylchroman-4-one eucomin from *Eucomis bicolor*) was presented by Dewick [42][43], who showed, by means of feeding experiments with isotopically labeled DL-phenylalanine-[1-^{14}C, L-phenylalanine-[U-^{14}C], sodium acetate-[2-^{14}C], and L-methionine-[*methyl*-^{14}C], that 2′-methoxychalcones are biosynthetic intermediate precursors of the homoisoflavonoids sappanin-, scillascillin-, and brazilin-type. The

results showed that the C_6-C_3 unit from phenylalanine was completely incorporated into the eucomin molecule, becoming the C-4, C-3, C-9, and the aromatic B ring, while the *O*-methyl on the B ring originated from methionine, which was also the source of C-2 (in the pyran ring C). The A-ring, on the other hand, was entirely from acetate. There is one difference from the biosynthesis of flavonoids—an additional atom derived from methionine, which is localized as a methyl group in the 2′-methoxychalcone molecule. The proposed mechanism for the formation of homoisoflavonoids from 2′-methoxychalcone included several oxidation steps necessary to activate the appropriate positions in the molecule for the chemical sense of a given transformation. However, what is missing from the literature is any indication of what processes, either enzymatic or abiotic, are responsible for the transformation of these chalcones into the corresponding homoisoflavonoids. Nevertheless, Dewick suggested that the formation of the 3-*spiro*-cyclobutene ring in scillascillin-type homoisoflavonoids may be related to the transformation of the corresponding sappanin-type homoisoflavonoid [43] (Scheme 5).



Scheme 5. Hypothetical biosynthetic routes to scillascillin-type homoisoflavonoids.

3. Biological Activities of Spiro-Flavonoids

In recent years, interest in spiro-compounds as drug candidates has increased [4][44][45][46]. Spiro-derivatives were shown to exert a broad range of biological activities, such as antioxidant, anti-inflammatory, neuroprotective, antimicrobial, antidiabetic, anticancer/cytotoxic, antiviral, antimalarial, etc. [4][40][44][46][47][48]. Their pharmacological potential is attributed directly to their inherent three-dimensionality, versatility, rigidity of spiro-cyclic scaffolds, good balance between flexibility and conformational restriction, and structural similarity to important pharmacophore centers [44][47][49]. Spiro-scaffolds are often developed successfully as drug candidates compared to compounds with too many flat aromatic rings (heterocycles) [44][47][49]. However, there is a gap in the synthesis of diverse, architecturally complex spiro-flavonoids. Therefore, the biological activities described here refer to compounds of natural origin.

3.1. Antioxidant Activity

The antioxidant capacity of the spiro-biflavonoid larixinol (**1**) originating from *Larix decidua* bark was evaluated in vitro using the DPPH assay (2,2-diphenyl-1-picrylhydrazyl) [50]. Compared to reference substances, larixinol was three times less active. Piacente et al. (2004) and Bassarello et al. (2007) tested the ability of **1** and yuccaols A-E (**36**–**40**), yuccaone A (**29**) from *Yucca schidigera* bark, and gloriosaols A-E (**44**–**48**) from *Y. gloriosa* root to scavenge the $ABTS^{\bullet+}$ radical (2,2′-azino-bis(3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt) [51][52]. Their activity was expressed as Trolox Equivalent Antioxidant Capacity (TEAC) values and compared to quercetin (positive control) (**Table 3**). Comp. **44**–**46**, and a mixture of gloriosaol D and E (**47** and **48**), showed the best antioxidant capacity in this study, much higher than quercetin [51]. Moderate $ABTS^{\bullet+}$ scavenging was observed for the dimeric spiro-flavonoids studied, with activity decreasing in the following order: yuccaol E, larixinol, yuccaol C, yuccaol D, and the weakest for yuccaol A and B, and yuccaone A (**40**, **1**, **38**, **39**, **36**, **37**, **29**, respectively) [52]. Compounds **36**–**40** in β-carotene/linoleic acid autoxidation assay showed significant activity, greater than the positive control—2,6-di-*tert*-butyl-4-methoxyphenol at 120 min [52]. Isolates **1**, **29** were not as active as positive control in this assay [52].

The 15-LOX (lipoxygenase) inhibition assay was used to test the ability of **37** and **44** isolated from the bark of *Y. schidigera* to protect polyunsaturated acids from peroxidation [40]. Their antioxidant activity was higher (EC_{50} = 9.66 µg/mL for yuccaol B, EC_{50} = 12.34 µg/mL for gloriosaol A) compared to the positive control: ascorbic acid (EC_{50} = 21.52 µg/mL) [40].

Nishida et al. (2013) [53] investigated antioxidant activities in DPPH (Trolox, curcumin, and α-tocopherol as positive controls), hydrogen peroxide (H_2O_2; Trolox as a positive control), and nitric oxide (NO; Trolox and curcumin as positive controls) scavenging assays of scillascillin-type homoisoflavonoids **49**, **51**, **52**, and **59** isolated from bulbs of *Scilla scilloides*. Isomuscomosin (**52**) tested at 500 µM showed more than 90% DPPH radical scavenging activity (EC_{50} = 22.9 µM; the other compounds tested were below 40%). In the H_2O_2 assay, scillascillin (**49**) and 2-hydroxy-scillascillin (**51**) showed activity below 40%, while isomuscomosin (**52**) and scillavone A (**59**) were highly active at 99.3% and 85.4%, respectively. In NO assays, the compounds tested were inactive (below 40% inhibition).

3.2. Anti-Inflammatory Activity

Li et al. (2009) investigated the ability of spiro-biflavonoids (**1–3**) to reduce the level of NO production in macrophage cell line RAW 264.7, induced by lipopolysaccharide (LPS) [54]. Only larixinol showed NO inhibitory activity at 100 µg/mL with and IC_{50} value of 60.0 µg/mL [54]. Daphnodorin C and I (**22**, **24**) and 2″-methoxy-daphnodorin C and 2″-methoxy-2-*epi*-daphnodorin C (**26**, **27**) were tested for inhibitory activity against LPS-induced NO production in RAW 264.7 macrophages [55][56]. Only **26** and **27** showed statistically significant inhibitory activity of 32% and 58%, respectively, against an increase of NO at a concentration of 100 µg/mL (compared to 50% for the positive control, aminoguanidine at 25 µM). Compounds **22** and **24**, isolated from the stems of *Daphne kiusiana* Miq., were investigated for their anti-inflammatory potential in the treatment of chronic obstructive pulmonary disease [57]. Comp. **22** most effectively suppressed the inflammatory response in both in vitro (phorbol 12-myristate 13-acetate)-stimulated human lung epithelial cells NCI-H292 and in an in vivo chronic

obstructive pulmonary disease model, using mice exposed to cigarette smoke and LPS. Daphnodorin C (**22**) negatively affected the expression of inflammatory genes by inhibiting nuclear factor kappa light chain enhancer of activated B cells (NF-κB) and specific MAPK signaling pathways (mitogen-activated protein kinases) (JNK and p38) and suppressed reactive oxygen species (ROS) products in vitro and in vivo. Daphnodorin C at 20 mg/kg was comparable to the positive control roflumilast at 5 mg/kg [57].

A series of spiro-flavostilbenoids (yuccaols A-E, **36**–**40**) from *Y. schidigera* bark was tested for anti-inflammatory activity using in vitro assays of COX-1, COX-2, and LTB_4 (leukotriene B4) formation mediated by 5-LOX. Comp. **36** and **37**, containing resveratrol as a stilbenic moiety, showed the highest inhibition against COX-1 and moderate inhibition against COX-2 [58]. Comp. **40**, which has a THMS moiety in its structure, expressed a slightly lower COX-1 and COX-2 inhibitory activity than comp. **36** and **37**. The investigated spiro-flavostilbenoids did not inhibit the formation of LTB_4 [58]. In another in vitro study, yuccaols A-C (**36**–**38**) were tested on the J774.A1 murine macrophage cells activated with *Escherichia coli* LPS [59]. The anti-inflammatory effect was observed only for compounds **36** and **38** when they were added 1 h before LPS stimulation. Yuccaol C showed the highest activity. It significantly inhibited the iNOS (inducible nitric oxide synthase) protein and NO formation through NF-κB deactivation in a dose-dependent manner, while yuccaol A inhibited significantly only NO release at the highest concentration [59]. Spiro-flavostilbenoids **38**–**43** were evaluated for anti-inflammatory activity on the mouse macrophage cell line RAW 264.7 [60]. Cells were preincubated with the compounds at 100 μM for 1 h and then stimulated with LPS. Yuccalide B (**42**), yuccaol C (**38**), and yuccaol E (**40**), which had the same relative configuration, effectively suppressed the mRNA level of iNOS. Yuccaols C-E significantly reduced transcription of the inflammatory cytokines IL-6 and IL-1β [60].

Waller et al. (2013) tested a series of homoisoflavonoids (at 10 μM) isolated from *Ledebouria socialis* and *L. ovatifolia*, including socialinone (**63**) and C-2 acetylated forms of 2-hydroxy-7-*O*-methyl-scillascillin (**50**) for the inhibitory activity of COX-1 (SC-560 as a positive control) and COX-2 (DuP-607 as a positive control) [61]. These compounds were found to be inactive in COX-2 assay. Nishida et al. (2014) [62] investigated the anti-inflammatory properties of scillascillin-type homoisoflavonoids from bulbs of *Scilla scilloides*, **49**, **51**, **52**, and **59**, using lipoxygenase (nordihydroguaiaretic acid as a positive control) and hyaluronidase (tannic acid as a positive control) as a model of in vitro inflammation. However, the compounds were not active at any of the concentrations tested (500, 750, and 1000 μM). Furthermore, the authors tested the anti-inflammatory effect of scillascillin-type homoisoflavonoids on LPS-activated RAW 264.7 mouse macrophages. Compound **59** showed weak inhibitory activity at 10 μM, while **49**, **51**, **52**, and **59** inhibited NO production level up to 50% at 50 μM. In another work [63], isomuscomosin (**52**) and compound **56** showed similar moderate anti-inflammatory activity in a microsomal fraction assay, while scillascillin (**49**) showed low activity (at concentrations of 250 μg/mL against indomethacin as a positive control). These compounds were found to be inactive in the COX-1 and COX-2 assays. Protosappanin D (**65**) and other constituents of *Caesalpinia sappan* were investigated using an in vitro assay with the J774.1 cell line [64]. Their inhibitory effects on NO and prostaglandin E_2 (PGE_2), as well as their suppressive effects on the expression of tumor necrosis factor-α (TNF-α), IL-6, COX-2, and iNOS mRNA level, were evaluated. As a result, **65** inhibited both NO (IC_{50} = 9.6 μM) and PGE_2 (IC_{50} = 7.8 μM) production. It showed the strongest suppression of

TNF-α (IC_{50} = 14.2 μM), IL-6 (IC_{50} = 3.0 μM), COX-2 (IC_{50} = 21.4 μM), and iNOS mRNA expression (IC_{50} = 13.2 μM) among the substances tested.

3.3. Neuroprotective Activity

Spiro-flavostilbenoids (**36**–**41**, **44**, **46**–**48**) and spiro-biflavonoids (**6**, **7**) from the bark of *Y. schidigera* were tested in vitro for their inhibitory activity against cholinesterases [7][65]. Acetylcholinesterase (AChE) from electric eel and butyrylcholinesterase (BChE) from horse serum were used in a modified Ellman spectrophotometric assay [7][65]. Compared to galantamine (positive control), the compounds tested showed moderate or weak inhibition of AChE. However, yuccaol B (**37**) and gloriosaol A (**44**) were the most potent inhibitors of BChE, with IC_{50} lower than the positive control (81.3 μM for **37**, 64.9 μM for **44**, and 124.0 μM for galantamine) [7]. Among yuccaols, the (2*S*,3*S*) diastereoisomers showed the highest anticholinesterase activity. The molecular interactions between the most active compounds (**37**, **44**) and human AChE/BChE were studied in silico. These compounds interacted similarly with the peripheral anionic site of AChE and were located deep in the catalytic site of BChE [7]. The neuroprotective effect of yuccaol B and gloriosaol A was also confirmed in vivo using adult zebrafish models [40]. The Y-maze test was used to assess the function of spatial working memory function, while the novel tank diving test was used to measure anxiety in *Danio rerio*. Spiro-flavostilbenoids **37** and **44** at doses of 1, 3, and 5 μg/L attenuated scopolamine-induced amnesia and anxiety, and improved precognitive and anxiolytic activities, to the level of the control group (untreated with scopolamine) [40].

3.4. Anticancer and Antitumor Activity

Spiro-biflavonoids abiesinols A-F (**1**, **4**, **12**–**15**), derived from the bark of *Abies sachalinensis*, were screened in vitro for antitumor-initiating activity, and then one of the most active compounds (**12**) was tested in vivo for a skin cancer chemopreventive effect [66]. Their inhibitory effect on the activation of NOR 1 ((±)-(*E*)-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-hex-3-enamide), a donor of nitric oxide, was evaluated in *Chang* human liver cells, using curcumin as a reference compound. The inhibitory activity of all compounds tested was similar to that of curcumin; only abiesinols E (**1**) and F (**4**), which have fewer OH groups in their structure than abiesinols A-D, were slightly less active. A two-step skin carcinogenesis assay was performed using specific-pathogen-free female ICR mice (6 weeks old). Abiesinol A (**12**) was administered orally (0.0025% of body weight) for 2 weeks; skin carcinogenesis was induced with a single dose of peroxynitrite ($ONOO^-$) and promoted with 12-*O*-tetradecanoylphorbol-13-acetate applied topically twice a week for 20 weeks [66]. Compound **12** showed significant antitumor-initiating activity in the in vivo test, the percentage of papilloma bearing mice was reduced to 33% at week 11, tumor formation was delayed by 2 weeks, and at the end of the experiment the average number of papillomas per mouse was reduced by a factor of 2, compared to the control group (no abiesinol A treatment) [66]. In contrast, the spiro-biflavonoids 3-*epi*-larixinol (**2**) and fragranols B and C (**8**, **9**), as well as the spiro-triflavonoid fragranol A (**30**), were not active against the human pancreatic cancer cell line PANC-1 in an in vitro assay. Compounds **2**, **8**, **9**, and **30** did not kill tumor cells at the maximum tested amount of 100 μM [34][35]. Spiro-biflavonoid genkwanol A (**23**), isolated from the aerial part of *Fumana procumbens* (Dunal) Gren. & Godr., was evaluated in vitro for its anticancer activity against A549 (adenocarcinomic human basal alveolar epithelial cell

line), MCF-7 (human breast cancer cell line), HeLa (human cervical cancer cell line), and BEAS-2B (human bronchial epithelial cell line) cells using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay [67]. Compound **23**, at the highest concentration tested (20 µg/mL), was active only against A549 cells, showing a 16.8% inhibition of cell viability [67]. Similarly, **23** had a moderate antimitotic effect, compared to the positive controls of colchicine and vinblastine in the microtubule polymerization bioassay in vitro [68]. In another work, the affinity of **23**, daphnodorin I (**24**), 2″-hydroxygenkwanol A (**28**), and 4′-methylgenkwanol A (**25**) to Hsp90 protein, one of the most promising targets for anticancer therapy, was tested in vitro [69]. All spiro-biflavonoids interacted with the immobilized protein, but comp. **28** was the most efficient [69].

Spiro-flavostilbenoids **36**–**38** inhibited Kaposi sarcoma (KS) cell proliferation, migration, and synthesis of the inflammatory mediator PAF (platelet-activating factor) in vitro [70]. Yuccaol C (**38**) was the most active, and it completely blocked the growth of the VEGF (vascular endothelial growth factor)-induced cells, more efficiently attenuated p38 and p42/44 MAP kinase signaling pathways activated by VEGF, and completely suppressed cell migration of KS cells after the PAF treatment. Moreover, yuccaols A-C inactivated VEGF-induced PAF biosynthesis through acetyl transferase blockade and enhanced PAF degradation through lysophospholipid transacetylase activation [70]. The cytostatic and pro-apoptotic activities of gloriosaols A-C (**44**–**46**) were tested in vitro against different cancer cell lines: MCF7 (breast carcinoma), HepG2 (hepatoblastoma), U937 (monocytic leukemia), Molt4 (lymphoblastic leukemia), and Jurkat (T-cell leukemia) [71]. Inhibition of cancer cell growth was observed after 24 h treatment with increasing concentrations of **44**–**46**. These stereoisomers showed different antiproliferative potentials: **46** had the lowest EC_{50} values, and the best effect was observed towards the U937 cell line, followed by **44** and **45** [71]. At the highest doses of 10–25 µM, gloriosaol C (**46**) induced apoptosis in the most sensitive cell line, U937, and tended to switch to necrosis at doses above 30 µM. However, the cytotoxic (proapoptotic) effect of **46** against human peripheral blood mononuclear cells was distinctly weaker. Gloriosaol C-induced apoptosis at doses > 10 µM caused mitochondrial depolarization and cytochrome *c* release in U937 cells [71]. The **44**–**46** tests altered the intracellular redox balance by increasing ROS (reactive oxygen species) release in U937 cancer cells exposed to these compounds for 1 h, and reducing ROS levels in cells simultaneously exposed to a pro-oxidant agent; that is, *t*-butyl hydroperoxide. The best pro-oxidant and antioxidant effect was observed for **46** at doses higher than 10 µM. This is one of the mechanisms by which these stereoisomers (**44**–**46**) were able to induce apoptosis in U937 cells. [71].

Schwikkard et al. (2019) [72] reported the antiproliferative activity of several structurally diverse homoisoflavonoids (including **49**, **52**, **53**, **63**, and **64**) from *Ledebouria ovatifolia* and *Chionodoxa luciliae* Boiss. against endothelial tumor cells (human retinal microvascular endothelial cells) and ocular tumor cells (uveal melanoma 92-1 and retinoblastoma Y79). The authors indicate that the activity of homoisoflavonoids tested was generally related to the substitution pattern of their A and B rings. However, the presence of the spiro-ring rendered the tested scillascillin-type homoisoflavonoids completely inactive, regardless of the substituents in the A and B rings. Likewise, Matsuo et al. (2014) [73] evaluated (3*R*)-5,7-dihydroxy-6-methyl-3-(3′-hydroxy-4′-methoxybenzyl)chroman-4-one and compound **57** (isolated from *Bessera elegans*), which differed only in the presence of the 3-*spiro*-cyclobutene ring (formed by the coupling of C-3 and C-2′ carbons), for cytotoxicity against human HL-60 promyelocytic leukemia cells and normal human diploid TIG-3 fibroblasts. The homoisoflavonoid without the cyclobutene ring showed a

potent tumor-selective cytotoxic activity against HL-60 cells, while **57** was noncytotoxic (and noncytotoxic against TIG-3). On the other hand, Chinthala et al. (2014) [74] performed an in vitro anticancer assay using scillascillin (**49**) isolated from *Ledebouria hyderabadensis* against the human cancer cell lines MCF-7 (breast cancer) and DU-145 (prostate cancer). Scillascillin showed significant activity (IC_{50} of 9.59 ug/mL and 11.32 µg/mL, respectively). Doxorubicin was used as a positive control. Its IC_{50} against the tested cell lines was 1.86 and 13.71 µg/mL, respectively.

3.5. Cytotoxicity/Mutagenicity

The lack of cytotoxicity of spiro-biflavonoid larixinol (**1**) at a dose of 100 µg/mL was confirmed in the RAW264.7 macrophages using the MTT assay [54]. No cytotoxicity of daphnodorin C (**22**) and daphnodorin I (**24**) at concentrations of 2.5–20 µM was detected against NCI-H292 human lung epithelial cells using the Cell Counting Kit-8 assay [57]. Spiro-flavostilbenoids **36–38** (yuccaols A-C) showed no toxicity against J774.A1 murine monocyte/macrophage cells at doses of 0.1–100 µM in an MTT test [59]. These compounds, at doses of 10–500 µg, confirmed their non-toxicity and demonstrated their non-mutagenicity in *Salmonella typhimurium* strains TA97, TA98, TA100, and TA102 tested by the microsome test (Ames test) [75].

3.6. Antiplatelet Activity

Sakuma et al. (1998) [76] tested spiro-biflavonoid daphnodorin C (**22**) isolated from *Daphne odora* roots for the activities of 12-lipoxygenase (12-LOX) and cyclooxygenase (COX). 12-LOX converts arachidonic acid to 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid, which is subsequently reduced to 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). 12-HETE has been reported to induce platelet aggregation. COX, on the other hand, produces prostaglandin endoperoxides. These are converted by thromboxane synthase to 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT). Daphnodorin C (**22**) caused 34.1% inhibition of 12-HETE formation and 26.6% inhibition of HHT at a concentration of 100 µM. This makes **22** a dual inhibitor of 12-LOX and COX in platelets.

In vitro, the spiro-flavostilbenoids **36** and **38** yuccaols A and C showed an inhibitory effect on platelet aggregation induced by thrombin and ADP (adenosine diphosphate) in a dose-dependent manner. Pig blood platelets pretreated with the compounds **36** and **38** at their highest concentration (25 µg/mL) caused a suppression of thrombin-induced aggregation by about 50% [77].

3.7. Antidiabetic Activity

A series of spiro-biflavonoid diastereoisomers (**1**–**4**, **10**, **11**) isolated from the stem bark of *Glyptostrobus pensilis* were screened in vitro for their ability to inhibit human protein tyrosine phosphatase 1B (PTP1B). This enzyme negatively regulates insulin signaling or epidermal growth factor pathways [78]. Spiropensilisol A (**10**) showed the best activity, inhibiting the enzyme to the same extent as a positive control (oleanolic acid) with an IC_{50} =3.3 µM. The remaining compounds tested were also active, with an $IC_{50} \leq 17.1$ µM. Using a molecular modeling approach, compound **10** was shown to interact with the catalytic site of the PTP1B enzyme [78]. Daphnodorins C and I (**22**,

24) and spiro-tetraflavonoids edgechrins A, B, and D (**32**, **34**, **33**) isolated from *Edgeworthia chrysantha* Lindl. were tested for α-glucosidase inhibitory activity, using *p*-nitrophenyl α-D-glucoside as substrate [79]. All compounds significantly inhibited α-glucosidase, but dimeric compounds **32**–**34** had a stronger activity than **22** and **24** (IC_{50} 1.09, 0.96, 2.13 versus 4.00 and 19.0, respectively, with 73.6 μM for the positive control, acarbose).

3.8. Antibacterial, Antifungal, and Antiviral Activity

Inamori et al. (1987) [80] tested the antifungal activity of spiro-biflavonoid daphnodorin C (**22**) against pathogens including *Pyricularia oryzae*, *Rhizoctonia solani*, *Phytophora infestans*, *Botrytis cinerea*, *Puccinia recondita*, and *Erysiphe graminis*, and its insecticidal activity (against *Spodoptera litura*, *Nilaparvata lugens*, *Callosobruchus chinensis*, and *Tetranychus urticae*). It showed antifungal activity only against *P. oryzae* associated with *Oriza sativa* as a plant host. The leaves of rice plant were incubated with daphnodorin C for 3 d before being inoculated with fungal spores for 5 d. The protective value of **22** was 89–90% at a concentration of 200–500 ppm. The insecticidal activity was negligible. Genkwanol A (**23**) caused an in vitro morphological deformation of the phytopathogenic fungus responsible for rice blast—*P. oryzae.* Compared to antifungal controls, that is, griseofulvin and nocodazole, **23** had lower minimum morphological deformation concentration [68].

Daphnodorin C (**22**), isolated from the bark of *Dahpne odora* Thunb., showed moderate inhibition of HIV-1 replication in MT-4 cells [81]. Compared to a positive control, 2′,3′-dideoxycytidine- 5′-triphosphate, comp. **22** had a weak inhibitory effect on HIV-1 reverse transcriptase. The authors suggest that daphnodorin C has anti-HIV-1 effects through inhibition of the early stage of viral replication [81]. Genkwanol A (**23**), derived from the root of *Wikstroemia indica* (L.) C.A.Mey., showed moderate activity against human immunodeficiency virus type 1 (HIV-1) in T4 lymphocytes (CEM cell line) [68].

Antibacterial activity of scillascillin-type homoisoflavonoids **49**, **52**, and **56** isolated from *Drimiopsis maculata* Lindl. & Paxton, and *Eucomis schijffii* Reyneke, was screened against *Staphylococcus aureus*, using the bioautographic and microplate assays [82]. Significant inhibitory activity was obtained for scillascillin (**49**), with a minimum inhibitory concentration (MIC) value of 0.50 mM (neomycin, positive control, had MIC = 0.0025 mM), while compound **52** (isomuscomosin) exhibited bacteriostatic activity with a bacteriostatic concentration value of 1.97 mM.

3.9. Phytotoxic Activity

Spiro-biflavonoid genkwanol A (**23**), isolated from the roots of *Stellera chamaejasme* L., showed strong phytotoxic activity against *Arabidopsis thaliana* seedlings, although only at relatively high concentrations (200 μg/mL), with an IC_{50} of 74.8 μg/mL, by inhibiting root growth [83]. This was confirmed by measuring the level of endogenous auxin in the root tip of the transgenic *A. thaliana DR5::GUS* line. This level was significantly reduced by **23** as a result of inhibition of auxin transport. At the same time, comp. **23** was not secreted into the soil by the roots of *S. chamaejasme*. Therefore, it is unlikely that it is responsible for the allelopathic effect exerted by this plant in nature.

3.10. Other Activities

Angiotensin II plays a key role in regulating blood pressure, and Takai et al. (1999) [84] tested the inhibition of human chymase-dependent angiotensin II-forming activity by daphnodorin A, daphnodorin B, and daphnodorin C (**22**) isolated from *D. odora*. The compound did not inhibit chymase-generated angiotensin II formation, but also did not affect the formation of angiotensin-converting enzyme-dependent angiotensin II and, unlike daphnodorin A, did not inhibit purified human tryptase.

The spiro-biflavonoids genkwanol A (**23**) and 2″-hydroxygenkwanol A (**28**) were tested in vitro for poly(ADP-ribose) polymerase 1 (PARP-1) inhibitory activity [85]. PARP-1 is an enzyme involved in the pathogenesis of cancer, inflammation, diabetes, and neurodegenerative diseases. Compound **28** binds efficiently to the PARP-1 protein catalytic domain in the nicotine binding pocket. 2″-Hydroxygenkwanol A strongly inhibited PARP-1 activity at submicromolar concentrations at a level comparable to the positive control—3-aminobenzamide [85].

Fusi et al. (2010) [86] investigated the vasorelaxant effects of scillascillin-type homoisoflavonoids **49**, **52**, and **56** obtained from *D. maculata* and *E. schijffii* using rat aortic ring preparations. The authors reported that both 60 mM K^+ (K60) and phenylephrine-induced tonic contractions were inhibited, in a concentration-dependent manner, by all homoisoflavonoids tested. Compound **56** was found to be the most effective vasorelaxing agent. This was in part due to the activation of soluble guanylyl cyclase. Sasaki et al. (2010) [87] investigated the vasorelaxant activity of protosappanin D (**65**) isolated from *C. sappan* on the rat aorta and mesenteric artery. The authors reported that **65** exhibited vasorelaxing activity on both phenylephrine-preconstricted blood vessels and that its activity was independent of the aortic endothelium and dependent on the mesenteric artery endothelium. The involvement of NO and prostaglandin as endothelium-derived relaxing factors was demonstrated in further experiments with N^G-nitro-L-arginine and indomethacin.

4. Conclusions

Spiro-flavonoids, due to the presence of an unusual structural element such as spiro-carbon, are attracting increasing interest because of their chemical and biological properties. A total of 65 spiro-flavonoid structures which were monomeric, as well as bi-, tri-, and tetrameric, belonging to several groups differing in the type of polyphenolic units and the way they are combined, were isolated and characterized. Spiro-biflavonoids were the most abundant group, most frequently isolated from the families Pinaceae, Thymelaeaceae, Cupressaceae, and Pentaphylacaceae. In turn, the richest source of spiro-compounds (thirty-four structures) was the Asparagaceae family, from which all known scillascillin-type homoisoflavonoids and all spiro-flavostilbenoids were derived. Most of the oligomeric spiro-flavonoids were isolated from woody plant parts (twigs, bark, and roots), while monomeric scillascillin-type homoisoflavonoids were obtained from bulbs. Methods used to isolate them mainly included classical extraction by maceration at room temperature using pure organic solvents of relatively different polarity and their mixtures, most often with water. The subsequent separation steps also included classical separation techniques based on the difference in solubility in two immiscible liquids (liquid-liquid extraction) as well as the use of column liquid chromatography in normal and reversed-phase systems and gel filtration.

The relative and absolute configurations of the complex structures of spiro-flavonoids, frequently containing multiple chiral carbons (including spiro-carbons), have been determined by a number of spectroscopic techniques, including nuclear magnetic resonance (NMR), electronic circular dichroism (ECD), X-ray diffraction (XRD), and chemical methods using chiral derivatizing agents. NMR and XRD are the methods most commonly used to determine their relative configurations, although an increasing number of cases of the use of quantum mechanical (QM) calculations (e.g., modified DP4+ probability method) are reported in the literature. Empirical methods have been used to assign absolute configuration, including the comparison of Cotton effects between known and newly described compounds. However, this method seems to be far from sufficient for structures with more than one chirality center, so it is necessary to systematically and correctly use QM techniques to predict ECD spectra using time-dependent density-functional theory calculations.

The potential health benefits of spiro-flavonoids have been summarized and the available results indicate significant anti-inflammatory, neuroprotective, antitumor/anticancer, and antidiabetic properties in vitro and in vivo of some spiro-biflavonoids and spiro-flavostilbenoids. On the other hand, scillascillin-type homoisoflavonoids showed good vasorelaxant activity. Therefore, future research should focus on these aspects of their activity.

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