

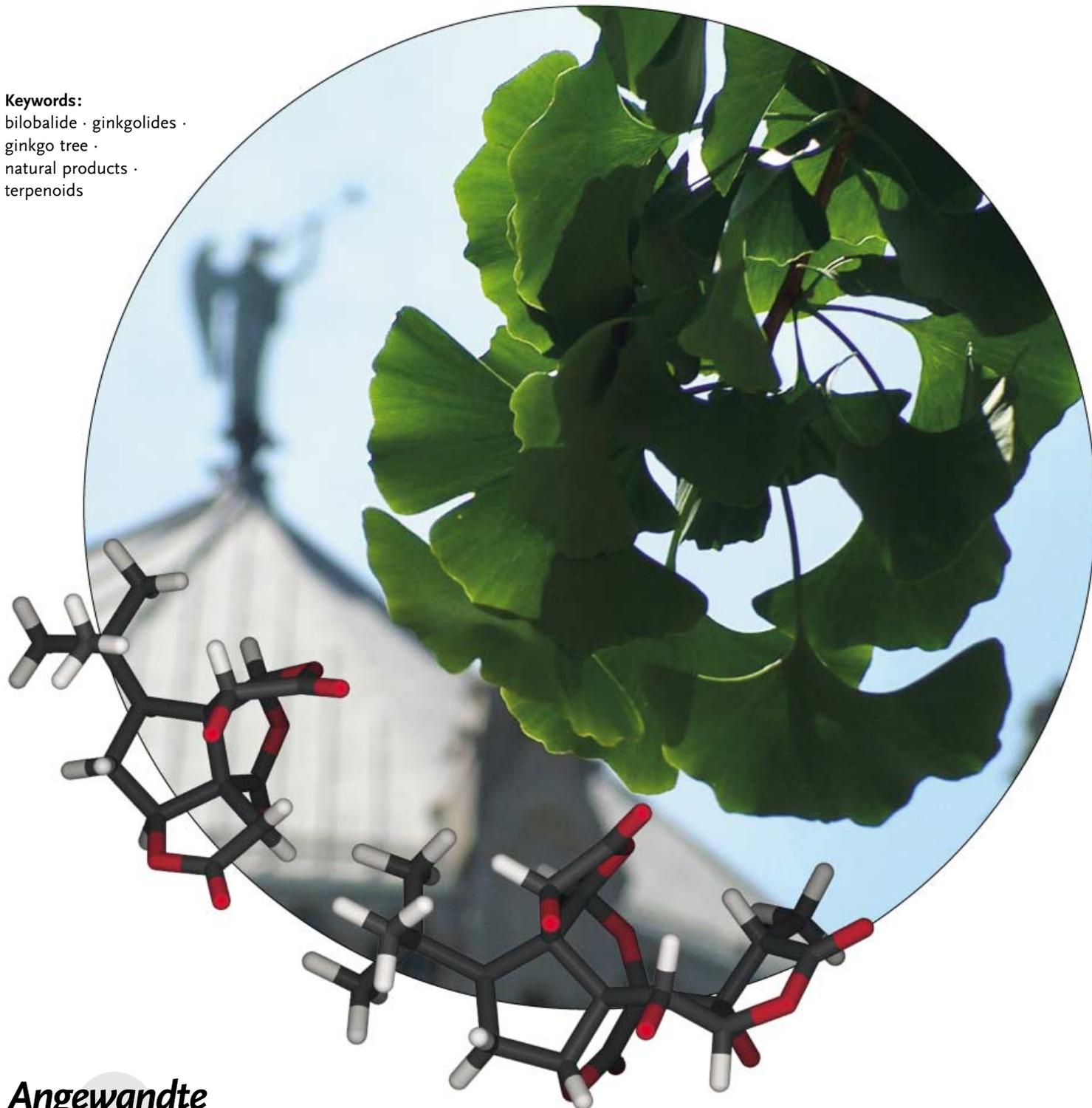
Natural Products Chemistry

Chemistry and Biology of Terpene Trilactones from *Ginkgo Biloba*

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Angewandte
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Ginkgo biloba, the ginkgo tree, is the oldest living tree, with a long history of use in traditional Chinese medicine. In recent years, the leaf extracts have been widely sold as phytomedicine in Europe and as a dietary supplement worldwide. Effects of Ginkgo biloba extracts have been postulated to include improvement of memory, increased blood circulation, as well as beneficial effects to sufferers of Alzheimer's disease. The most unique components of the extracts are the terpene trilactones, that is, ginkgolides and bilobalide. These structurally complex molecules have been attractive targets for total synthesis. Terpene trilactones are believed to be partly responsible for the neuromodulatory properties of Ginkgo biloba extracts, and several biological effects of the terpene trilactones have been discovered in recent years, making them attractive pharmacological tools that could provide insight into the effects of Ginkgo biloba extracts.

1. Introduction

Since the discovery of the ginkgolides and bilobalide, termed terpene trilactones (TTLs), from *Ginkgo biloba* in 1932, the elucidation of their unique structures 35 years later, and the discovery of their ability to antagonize the platelet-activating factor receptor (PAFR) in 1985, TTLs have attracted intense interest, which is reflected in the literature related to *G. biloba*,^[1,2] *G. biloba* extracts,^[3] and TTLs.^[4] Two reviews on the chemistry and pharmacology of ginkgolides have also been published, the latest in 1991,^[5,6] hence this Review focuses primarily on developments in the chemistry and (neuro)biology of TTLs since 1991. This includes discovery of a new and general biosynthetic route that originated from biosynthetic studies of ginkgolides, total syntheses, intriguing chemical reactivities, extensive structure–activity relationship studies on the PAFR, recent findings that ginkgolides halt β -amyloid formation, and the discovery of a new target for ginkgolides.

1.1. The Ginkgo Tree

The Ginkgo tree (*Ginkgo biloba* L.; from Japanese *ginkyo*, meaning silver apricot) or maidenhair tree (Figure 1), is characterized by fan-shaped leaves and fleshy, yellow, foul-smelling seeds, enclosing a silvery, edible inner kernel. *G. biloba* is the only surviving member of a family of trees, *Ginkgoaceae*, which appeared in the Jurassic period 170 million years ago, and for this reason is called the “living fossil.” There is a gap of 100 million years between the current and ancient tree in the fossil record but recent paleontological findings of a fossil over 121 million years old have shown that the tree has barely changed since the days of the dinosaurs.^[7] It is distinct from all other living plants and is often categorized in its own division, Ginkgophyta, by botanical taxonomists. *G. biloba* is believed to be the oldest living tree species^[8] and is capable of reaching ages in excess of one thousand years.^[9] Recent investigations have used this “living fossil” to evaluate CO₂ levels in prehistoric times.^[10,11]

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G. biloba is dioecious, that is, the male and female structures exist on separate trees, but the two structures can only be distinguished after the tree is around 30 years old. Ginkgo trees can grow over 35 meters high, with the main



Figure 1. A *Ginkgo biloba* tree near Riverside Church, New York.

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stem up to 10 meters in girth. The tree is a resilient survivor in polluted environments, growing where other trees find it difficult, and is exceptionally resistant to attacks by fungi and insects. More than 100 years ago, the Japanese botanist Sakugoro Hirase reported the discovery of the motile sperm of *G. biloba*, which in terms of evolutionary plant history provided a critical connection between the life cycles of lower and higher plants.

G. biloba was grown throughout China and Korea, and was introduced into Japan about 800 years ago, then into Europe around 1730, and to North America in 1784. The term “Ginkgo” was first used by the German physician and botanist Engelbert Kaempfer in 1712, but Linnaeus provided the binominal terminology “*Ginkgo biloba*” in 1771, “biloba” meaning two-lobed (from Latin *bi*: double, *loba*: lobes), referring to the fan-shaped leaves split in the middle (Figure 2). The shape of the leaves inspired German poet, scientist, botanist, and philosopher Johann Wolfgang von Goethe (1749–1832) to write a poem called “Ginkgo biloba” dedicated to his former lover Marianne von Willemer.^[12] The



Figure 2. Leaves and nuts from *Ginkgo biloba*.

young Ginkgo leaf symbolizes Goethe's theme: the individual and the pair as indistinguishable beings.

The earliest records on the use of *G. biloba* as medicine dates back to the book of Liu Wen-Tai in 1505 A.D.^[13] As described in *Chinese Materia Medica* by Pen Tsao Ching (1578), *G. biloba* was used to treat aging members of the royal court for senility. Although leaf preparations are the primary source of *G. biloba* today, it was the fruits that were described in these ancient Chinese medical records. Today, Ginkgo nuts (Figure 2) are used in Japanese and Chinese cuisine, either grilled or boiled. Care must be taken as excessive intake of these nuts can cause food poisoning, presumably due to their content of 4-*O*-methylpyridoxine, which is a known convulsive agent through the inhibition of γ -aminobutyric acid (GABA) synthesis.^[14,15]

1.2. Ginkgo Biloba Extract

Leaf preparations of *G. biloba* were introduced to the Western world around 1965 by the German company Dr. Willmar Schwabe^[13] under the trade name Tebonin. Later, the Schwabe company established a collaboration with the French company Beaufour-Ipsen, and together they developed a standardized the *G. biloba* extract, which they termed EGb 761^[16] and is sold under trade names such as Tanakan, Rökan and Tebonin forte. Since then, a wealth of *G. biloba* products have entered the market, and *G. biloba* extract is now among the best-selling herbal medications worldwide. In 1998, Americans spent approximately 4 billion dollars on botanical medicines, and *G. biloba* ranked first among herbal medications. Today over 50 million *G. biloba* trees are grown, especially in China, France and South Carolina, USA, which produce approximately 8000 tons of dried leaves each year.^[17]

The *G. biloba* extract EGb 761 is obtained from dried green leaves that are extracted with an acetone/water mixture. This extract has been standardized to contain 6% TTLs (3.1% ginkgolides and 2.9% bilobalide) and 24% flavonoids.^[13] The flavonoids are almost exclusively flavanol-*O*-glycosides, a combination of the phenolic aglycones quercetin, kaempferol, or isorhamnetin, with glucose or rhamnose or both at different positions of the flavonol moiety. Interestingly, although the bioavailability of flavonoids in *G. biloba* extract is controversial, it is generally assumed that flavonoids do not penetrate the blood–brain



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Koji Nakanishi was born in 1925 in Hong Kong. He received his BSc at the Nagoya Imperial University with F. Egami and Y. Hirata. After spending part of his graduate years with L. F. Fieser at Harvard, he received his PhD in 1954 from Nagoya University. He became Professor at Tokyo Kyoiku University in 1958, and later at Tohoku University, Sendai. In 1969 he moved to Columbia University, where he is currently Centennial Professor of chemistry. After determining the structure of the ginkgolides in 1967, he became engaged in investigations into their mode of action.

barrier (BBB), or at least not in sufficient quantities to exert physiological effects on the central nervous system (CNS). On the other hand, the bioavailability of ginkgolides and bilobalide was 70–80% after an 80 mg dose of EGb 761, with half-lives of 3–5 h.^[18] EGb 761 contains several other components, including proanthocyanidins (prodelphinidins) and organic acids. Moreover, the content of ginkgolic acids (anarcadic acids) in EGb 761 is limited to 5 ppm owing to the allergenic properties of these compounds.^[13]

EGb 761 remains the most widely used extract for scientific investigations of the pharmacological effects of *G. biloba*, and several recent reviews have described the effects of the extract on the CNS.^[18–22] The neuromodulatory effects of EGb 761 include improvement of cognition, antioxidant effects, increased cerebral blood flow and circulation, modification of neurotransmission, and protection against apoptosis.^[18] In Germany, the *G. biloba* extract is used for treatment of “disturbed performance in organic brain syndrome within the regimen of a therapeutic concept in cases of dementia syndromes with the following principal symptoms: memory deficits, disturbance in concentration, depressive emotional condition, dizziness, tinnitus, and headache.”^[23] EGb 761 is among the most prescribed medications in Germany and France for the treatment of dementia-related symptoms.^[24]

The most widely studied application of EGb 761 has been in the potential treatment of Alzheimer's disease (AD).^[25–27] In two pivotal studies, a total of 549 AD patients were evaluated for the effect of EGb 761.^[28,29] In both studies, EGb 761 significantly slowed the development of the cognitive symptoms of dementia, and regression on certain data points was delayed by 7.8 months, which is similar to the currently available AD treatments, Aricept (donepezil, 9.5 months) and Exelon (rivastigmine, 5.5 months), both acetylcholinesterase inhibitors. A recent follow-up study showed that EGb 761 had a better effect in very mild to mild cognitive impairment, rather than in severe dementia, suggesting that EGb 761 stabilizes or delays the onset of symptoms.^[30] Aside from the above-mentioned clinical trials, more than 40 clinical studies were performed with extracts of *G. biloba*,^[31–37] and virtually all trials reported positive results regarding various aspects of cerebral insufficiency, although with differing degrees of efficacy. The observed clinical effects could be explained by recent findings that EGb 761 can inhibit the formation of β -amyloid aggregation,^[38–40] which is believed to be highly important in the development of AD.^[41] Another study looked at the effect of EGb 761 on the modulation of gene expression in the cortex and hippocampus of mice, and found that several genes were affected, including genes that are believed to be involved in AD.^[42,43]

Recent studies on healthy adults using a computerized test battery have shown beneficial effects of the *G. biloba* extract on short-term memory;^[44] similar results were reported in three other studies.^[45–47] However, a recent study on healthy volunteers concluded that a 6-week treatment of 120 mg EGb 761 per day did not enhance the performance of memory or related cognitive functions.^[48] This was also the conclusion of a previous study, in which patients were treated with EGb 761 for 30 days.^[49] In contrast, a 23-patient pilot

study on the effects of EGb 761 on multiple sclerosis (MS) demonstrated improvement of cognitive and functional abilities in MS;^[50] a dose of 240 mg day⁻¹ of EGb 761 showed beneficial effects on attention, memory, and functioning after three months in patients with mild MS.

Therefore EGb 761 might be beneficial in relieving symptoms of AD, although the reported effects are often small. In light of the current lack of treatment for AD patients,^[41,51] EGb 761 could prove to be useful as an alternative to the currently available treatments. On the other hand, the postulated effects of EGb 761 in improving memory functions in healthy people are still controversial and not fully corroborated, despite numerous studies. Several suggestions have been made, for example, that EGb 761-induced changes in brain function may be associated with the prevention of age-related decreases in serotonin binding^[52] or α_2 adrenergic receptor density^[53] in cerebral membranes, but these studies have not been sufficiently validated.

Very little is known about which components of EGb 761 are efficacious, and thus the molecular basis for the action of *G. biloba* constituents on the CNS is poorly understood. The major components of the extract are flavonoids and terpene trilactones; the flavonoids are assumed not to penetrate the BBB, whereas nothing is known about whether terpene trilactones penetrate the BBB. However, it is generally assumed that the lipophilic character of TTLs renders these compounds permeable to the BBB, and therefore it appears that TTLs are partially responsible for the effects of *G. biloba* extracts on the CNS.

2. Isolation and Structure Elucidation

2.1. Structure Elucidation

A large number of *G. biloba* natural products have been identified, such as flavonoids and anarcadic acids, but the TTLs, that is, ginkgolides and bilobalide,^[54] are unique constituents of *G. biloba* and are found exclusively in the *G. biloba* tree. The ginkgolides are diterpenes with a cage skeleton consisting of six five-membered rings: a spiro[4.4]nonane carbocyclic ring, three lactones, and a tetrahydrofuran ring. Furthermore, they contain an unprecedented *tert*-butyl group. The ginkgolides vary only in the number and positions of their hydroxy groups (Figure 3). These compounds were first isolated from the root bark of *G. biloba* in 1932 by

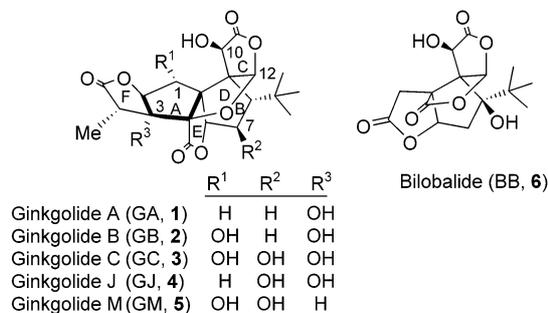


Figure 3. Structure of the five ginkgolides and bilobalide.

Furukawa.^[55] In 1967, Maruyama et al. succeeded in isolating ginkgolides A (GA; **1**), B (GB; **2**), C (GC; **3**), and M (GM; **5**) from the root bark and deduced their very unique structures (Figure 3).^[56–61] Independently, Okabe and co-workers determined the structures of GA (**1**), GB (**2**), and GC (**3**) from leaves of *G. Biloba* by means of X-ray crystallography.^[62,63] Another ginkgolide, ginkgolide J (GJ; **4**), was isolated from the leaves of *G. biloba* in 1987.^[64] Interestingly, it appears that GJ (**4**) is only found in the leaves, whereas GM (**5**) is found only in the root bark.

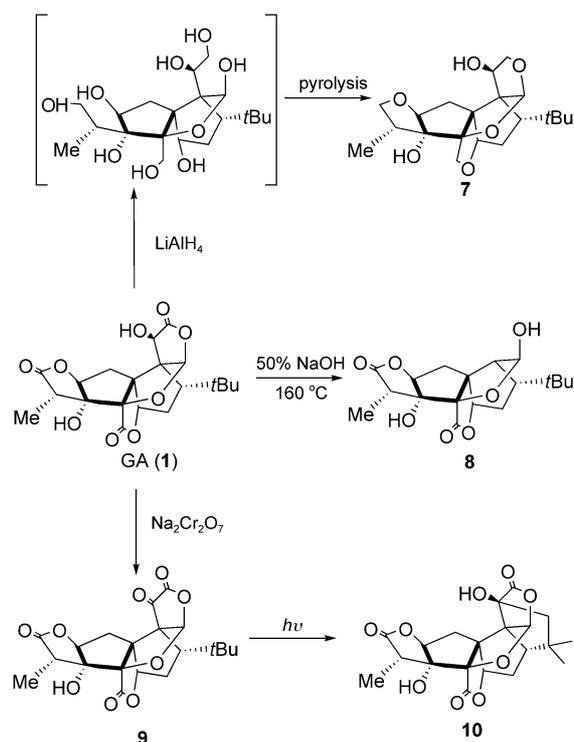
An account of the studies leading to the structures of GA (**1**), GB (**2**), GC (**3**), and GM (**5**) has been published elsewhere;^[65,66] however, a few highlights are worth mentioning. The structural studies by Maruyama et al. were facilitated when a typhoon hit Sendai, Japan, and permission was received to use the root bark from five felled trees (100 kg) in 1964. This gave, after extraction, chromatography, and fractional recrystallization, 10 g each of GA (**1**) and GB (**2**), 20 g GC (**3**), and 200 mg GM (**5**) (Figure 4).^[56] Purification of ginkgolides was seriously hampered by their remarkable



Figure 4. Polymorphic crystals of ginkgolide C obtained by fractional recrystallization (cm scale).

tendency to exhibit polymorphism and to form mixed crystals. Satisfactory results were achieved only after 10–15 repeated fractional crystallizations; the purity was checked by NMR spectroscopic analysis or optical rotation. The complex, but aesthetic structures depicted in Figure 3 were determined by performing extensive chemical reactions and spectroscopic studies on the native TTLs and approximately 70 derivatives.^[56–61]

The compound that played a key role in the structural studies was the GA “triether” (in fact a tetraether, **7**, Scheme 1), which was obtained by reduction of GA (**1**) with concomitant pyrolysis. The carbonyl groups of the three lactones were selectively reduced, while leaving the ginkgolide core untouched (Scheme 1).^[57] The NMR spectra of ginkgolides showed no connectivity in the proton systems, which are disconnected by intervening quaternary and carbonyl carbons atoms; however in the GA “triether” **7**, the three carbonyl groups are replaced by methylene units, and thus could be submitted to exhaustive double and triple decouplings. The assignments were corroborated by reduction



Scheme 1. Transformations of GA (**1**) used in the structure determination, demonstrating the remarkable stability of the ginkgolide skeleton.

of GA (**1**) with LiAlD_4 instead of LiAlH_4 . The studies also led to the observation of what is now known as the nuclear Overhauser effect (NOE), unknown at that time. Irradiation of the singlet assigned to the *tert*-butyl group led to integrated area enhancements of some protons related to the Overhauser effect known in electron spin resonance (ESR).^[60,67] It was during the course of these studies that Anet and Bourn described the first observation of an NOE.^[68]

The unique stability of the ginkgolide core was encountered when GA (**1**) was treated under alkali fusion conditions (50% NaOH, 160 °C, 30 min). Loss of two carbon centers as oxalic acid gave the hemiacetal bisnor-GA (**8**). Another example of this stability was found when an ice-cooled solution of GA (**1**) was treated with sodium dichromate in concentrated sulfuric acid, which simply oxidized the hydroxylactone to give oxolactone **9**. The latter compound was a source of confusion: The ^1H NMR of **9** showed the familiar nine-proton *tert*-butyl singlet, whereas signals from an α -ketolactone were not observed in the UV or CD spectra. The electronic data were measured with a sample left for some time, and it turned out that during this period an unexpected photocyclization of **9** to photodehydro-GA **10** (Scheme 1) had taken place. Similarly, remeasurement of the ^1H NMR spectrum showed that the *tert*-butyl group had disappeared and had been replaced by a geminal dimethyl group. These unusual spectroscopic and photochemical properties of dehydro-GA were subsequently clarified.^[69,70]

The absolute configuration of ginkgolides was determined based on the octant rule analysis of 7-oxo-GC 10-monomethyl ether and another ketone derivative by Maruyama et al.^[59]

Okabe and co-workers treated GA (**1**) with *p*-bromobenzoyl bromide to give prismatic crystals of 3-mono-*p*-bromobenzoate, which were submitted to crystallographic studies.^[63] Initially, there was a discrepancy in the reported structures of GB (**2**) and GC (**3**) with respect to the relative stereochemistry of the hydroxy group at C1, as Maruyama et al. concluded that the hydroxy group was in the α configuration,^[59] whereas Okabe and co-workers suggested it to be in the β configuration.^[62] Detailed NMR spectroscopic studies^[71] and X-ray crystal structures of GB (**2**) and GC (**3**)^[72,73] confirmed the α configuration of 1-OH. Subsequent X-ray crystal structures of GA (**1**), GB (**2**), and GC (**3**) revealed that the overall structures of these molecules are remarkably similar. However, there is a slight difference between the conformation of GA (**1**) and those of GB (**2**) and GC (**3**), owing to intramolecular hydrogen bonds in GB and GC, primarily between 1-OH and 10-OH, but also between 1-OH and 3-OH.^[72,73] Recently, more-accurate X-ray crystal structures of GA (**1**), GC (**3**), and GJ (**4**) obtained at 120 K^[74] showed that the overall conclusions regarding the structure of the ginkgolides were as previously reported.^[72,73]

A structure related to the ginkgolides, but with the empirical formula $C_{15}H_{18}O_8$ was isolated from the leaves of *G. biloba* by Major.^[8] A few years later Weinges and Bähr reported that the same compound, which they named bilobalide (BB, **6**, Figure 3), also contained the characteristic *tert*-butyl group, as well as a secondary and a tertiary hydroxy group.^[75] Through a collaborative effort by Major, Weinges, Nakanishi and co-workers, the structure was determined in 1971, showing that, like the ginkgolides, it contained three lactones and a *tert*-butyl group, but only one carbocycle.^[76,77] Bilobalide (**6**) is thus closely related to the ginkgolides and is the most abundant TTL in the EGb 761 extract. Weinges et al. obtained an X-ray crystal structure of BB (**6**) in 1987, which in combination with CD studies, was used to confirm the stereochemistry of the hydroxy groups.^[78]

2.2. Isolation and Quantification

In the original reports describing the isolation and structure elucidation of TTLs, several extractions of root bark or leaves, followed by chromatography, and 10–15 rounds of fractional recrystallization were required to obtain the individual TTLs in pure form. Subsequently, considerable effort was invested to simplify the isolation and quantification of TTLs, as recently reviewed by van Beek.^[79,80]

The first step in obtaining pure TTLs is extraction from the leaves. In most cases, various water-containing solvent systems such as water/acetone or water/methanol are used. Apolar constituents are excluded, whereas all of the TTLs are collected, including GB (**2**), which is only scarcely soluble in water. Basic extractions should be avoided owing to the instability of BB (**6**) in solutions with $pH > 7$.^[80] Recently, an improved extraction of TTLs that takes advantage of their stability under a variety of conditions, such as oxidation and heat, was developed.^[81] During this process, the leaves are boiled for 1 hour in dilute hydrogen peroxide, followed by

extraction with ethyl acetate to generate an off-white powder with a TTL content of 60–70%.^[81] The treatment with hydrogen peroxide removes the constituents that lead to extensive emulsions in the extraction steps, thus greatly shortening the process.

With a crude mixture of TTLs in hand, a true challenge is to separate the individual ginkgolides and bilobalide. Whereas bilobalide is relatively easily separated from the ginkgolides by using standard column chromatography, the separation of the individual ginkgolides is far from trivial. Not only do the ginkgolides differ only by one or two hydroxy groups, but in some cases these hydroxy groups are involved in hydrogen bonding (e.g. 1-OH and 10-OH, Figure 3), and therefore do not significantly alter the overall polarity of the molecule. A simple improvement in the separation was reported by van Beek and Lelyveld in which the hydrogen bonds are disrupted by using silica gel impregnated with sodium acetate. This technique is coupled with preparative-scale medium-pressure liquid chromatography (MPLC)^[82] and TLC detection of TTLs.^[83] In the purification step, the labile nature of BB (**6**) is also a concern, as it degrades on alumina columns.^[84] Quantitative 1H NMR spectroscopic analysis was used as a convenient method to determine the amount of TTLs in various preparations,^[85] since the signals for the 12-H of TTLs^[86] are distinct and well-separated, integration intensities of these signals are then compared to that of maleic acid (MA; Figure 5).^[87] This remains the best

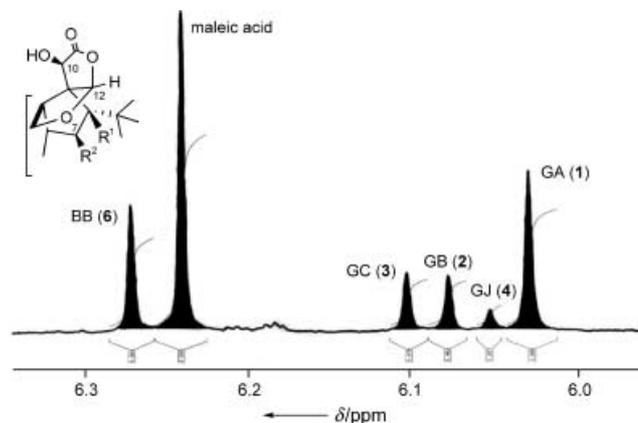


Figure 5. Quantitative 1H NMR spectroscopic analysis is an efficient and simple method to determine the amount of TTLs in a mixture.

method for determining the relative contents of various ginkgolides, as well as the purity of the isolated ginkgolides.

TTLs lack common chromophores and therefore UV detection is not suitable. Other detection methods, such as refractive index (RI), evaporation light-scattering detection (ELSD), and MS have been utilized. The first reported separation and quantification of TTLs, however, involved conventional HPLC/UV detection.^[88] RI detection was successfully applied later.^[89,90] Although UV detection is often preferred^[91] as it demonstrates better sensitivity than RI detection, the selectivity is superior when using RI.^[79] Several recent reports describe LC/MS to separate and quantify TTL content in various *G. biloba* extracts, as well as in plasma after

intake of *G. biloba* extract. The major difference in the detection systems lies in the MS procedures used, which could be electrospray ionization (ESI)^[92] or atmospheric-pressure chemical ionization (APCI).^[93,94] Recently, LC/MS (ESI) was used for analysis of commercial *G. biloba* products in which large variations in the composition and concentration of TTLs were observed.^[95] As an alternative to MS, ELSD was also successfully applied to quantify TTLs in *G. biloba* extracts.^[96,97]

3. Biosynthesis

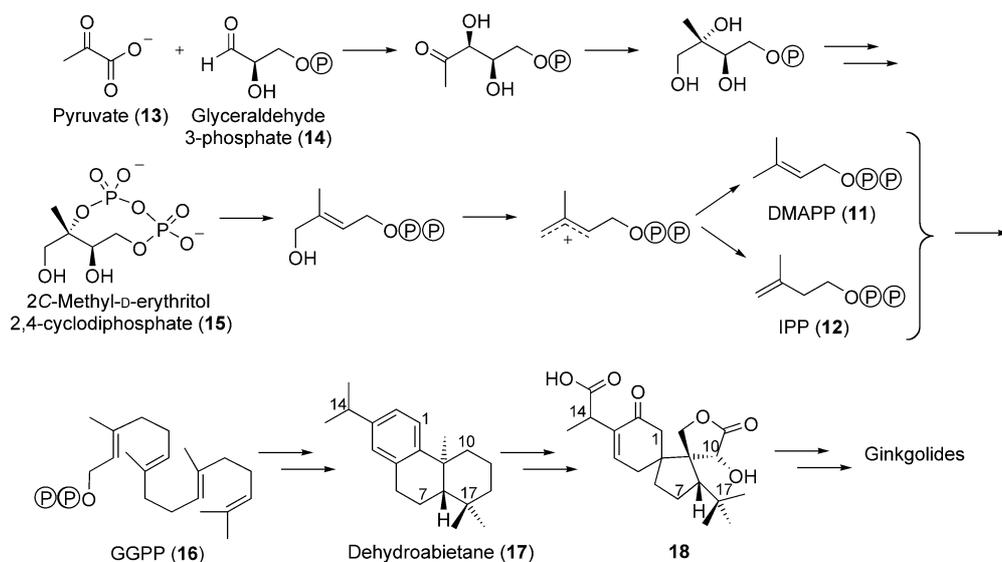
Because of their complex framework, it was not immediately clear to which structural category ginkgolides belong. Biosynthetic studies with [2-¹⁴C]acetate and D,L-[2-¹⁴C]mevalonate appeared to suggest that the overall terpenoid origin of ginkgolides involved the two conventional precursors, dimethylallyl pyrophosphate (Scheme 2, DMAPP, **11**) and isopentenyl pyrophosphate (IPP, **12**).^[98] Until recently, DMAPP and IPP were thought to be biosynthesized through the conventional mevalonate pathway, during which three molecules of acetyl coenzyme A react and are reduced to give mevalonic acid, which is then phosphorylated, followed by elimination of phosphate and CO₂ to give DMAPP and IPP. However, recent biosynthetic studies led to the surprising discovery of a non-mevalonate pathway. The earlier mevalonate pathway described by Nakanishi and Habaguchi^[98] turned out to be a minor pathway for ginkgolide biosynthesis.

Rohmer^[99] used ¹³C NMR spectroscopy as the major tool to show the surprising existence of an alternative path, the non-mevalonate or deoxyxylolose phosphate pathway, in which pyruvate (**13**) and glyceraldehyde 3-phosphate (**14**) react to produce 2C-methyl-D-erythritol 2,4-cyclodiphosphate (**15**), and ultimately DMAPP (**11**) and IPP (**12**) (Scheme 2).^[100,101] Independently, Arigoni and Schwarz^[102]

studied the biosynthesis of ginkgolides with a *G. biloba* embryo system and ¹³C-labeled glucose. They showed that ginkgolides were biosynthesized through the non-mevalonate pathway. These comprehensive biosynthetic studies led to the discovery of a novel metabolic pathway for ginkgolides that could be quite widespread. Initially, IPP and DMAPP react to produce the universal diterpene precursor geranylgeranyl pyrophosphate (GGPP, **16**), which is converted into a tricyclic intermediate, levopimaradiene. This leads to dehydroabietane (**17**), which is transported from the plastids into the cytoplasm. Compound **17** is then converted into the ginkgolides through a complex series of reactions involving several oxidation steps, probably with **18** as an intermediate (Scheme 2).^[102]

In another set of studies, ¹⁴C-labelled CO₂ was incorporated into ginkgolides and bilobalide, and the chronology of labeling indicated an in situ bioconversion of GA (**1**) into GC (**3**). Similar studies suggested dehydroabietane (**17**) as a biosynthetic precursor of TTLs.^[103,104] Labeling with [U-¹⁴C]glucose indicated that all the biosynthetic steps to the ginkgolides take place in the root, and the products are then translocated to the leaves.^[103] On the other hand, another study by Carrier et al. monitored the incubation of [1-¹⁴C]IPP into farnesyl pyrophosphate (FPP) and GGPP (**16**) and correlated this with the presence of TTLs. It was concluded that ginkgolides were synthesized in the aerial parts of the plant.^[105]

Recently, a cDNA that encodes *G. biloba* levopimaradiene synthase, a diterpene synthase involved in ginkgolide biosynthesis, was isolated and characterized.^[106] *G. biloba* levopimaradiene synthase is responsible for a multistep reaction sequence that converts GGPP (**16**) into levopimaradiene,^[106] a double-bond positional isomer of abietadiene isolated from *G. biloba* seedlings.^[104] The cloning and isolation of this enzyme, together with cloning of other biosynthetic genes, could provide a route for the large-scale production of ginkgolides.



Scheme 2. Biosynthesis of ginkgolides through the non-mevalonate pathway. $\textcircled{P} = \text{PO}_3^{2-}$.

4. Synthetic Studies

Since the discovery of their complex structures in 1967, TTLs have attracted great interest as targets for total synthesis, an area pioneered by Corey (Figure 6). The

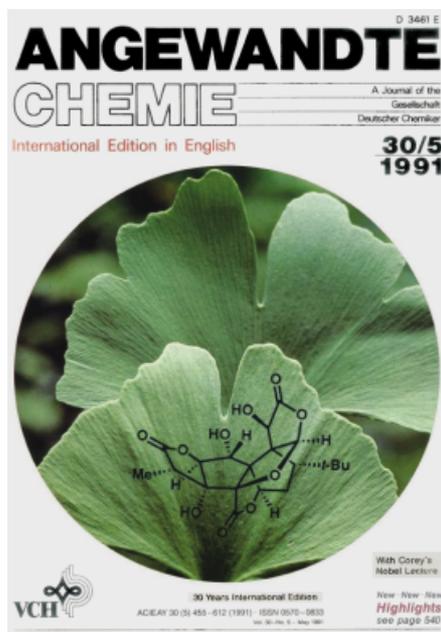


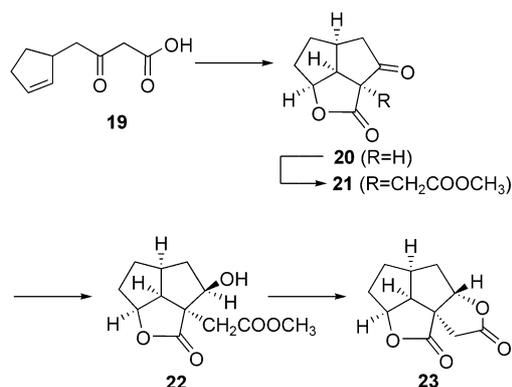
Figure 6. *Ginkgo biloba* leaves featured on the cover of *Angewandte Chemie*, International Edition in English (Issue 5, 1991) containing the Nobel lecture by E. J. Corey.

ginkgolide skeleton consists of six highly oxygenated rings, 10–12 stereogenic centers, and a unique *tert*-butyl group, thus providing a formidable synthetic challenge. When GB (**2**) was shown to be a potent antagonist of the platelet-activating factor (PAF) receptor, a large number of ginkgolide derivatives were prepared for structure–activity relationship (SAR) studies. Furthermore, numerous synthetic studies involved approaches to radiolabeled, acetylated, and glycosylated ginkgolides and ginkgolides with a rearranged skeleton. NMR titration studies of ginkgolides were used to determine their pK_a values.

4.1. Total Syntheses

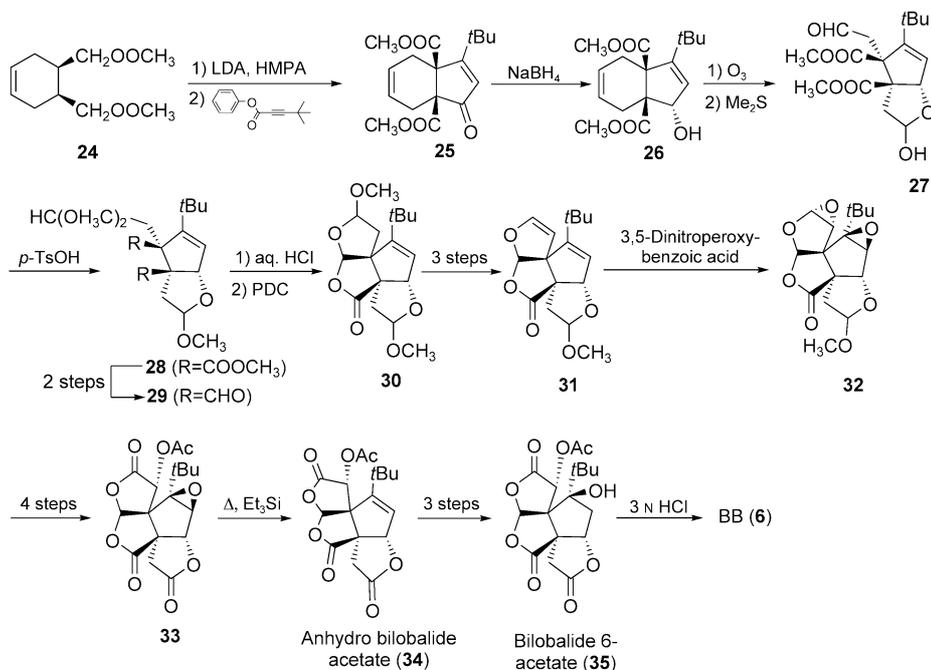
4.1.1. Bilabolide

In 1984 Corey and Kang reported the first progress towards the total synthesis of bilabolide (BB, **6**, Figure 3). They described a general approach to the synthesis of polycyclic γ -lactones, as illustrated with the synthesis of dilactone **23** from ketoacid **19** (Scheme 3).^[107] Corey and Su then



Scheme 3. A general method for the synthesis of polycyclic γ -lactones.

reported the total synthesis of bilabolide in 1987.^[108] Bicyclic ketone **25**, which contains all the carbon atoms of bilabolide was prepared by generating the dianion of **24** with lithium diisopropylamide (LDA) and hexamethylphosphoramide (HMPA), followed by treatment with phenyl 3-*tert*-butylpropionate in a previously developed annulation reaction (Scheme 4).^[109] Reduction of **25** with sodium borohydride afforded **26**, which was converted into lactol **27** by ozonolysis and reduction. This product was treated with *p*-toluenesulfonic acid (*p*-TsOH) to give **28**, which in two steps was converted into dialdehyde **29** by reduction with lithium aluminum borohydride and subsequent Swern oxidation. Treatment of **29** with dilute hydrochloric acid gave epimeric lactols, which were oxidized with pyridinium dichromate (PDC) to give **30**. Exposure of **30** to aqueous potassium hydroxide, THF, and ethanol led to a remarkably selective replacement of the methoxy substituent with a hydroxy group, followed by chlorination and elimination to give **31**. The two



Scheme 4. Total synthesis of BB (**6**) by Corey et al.

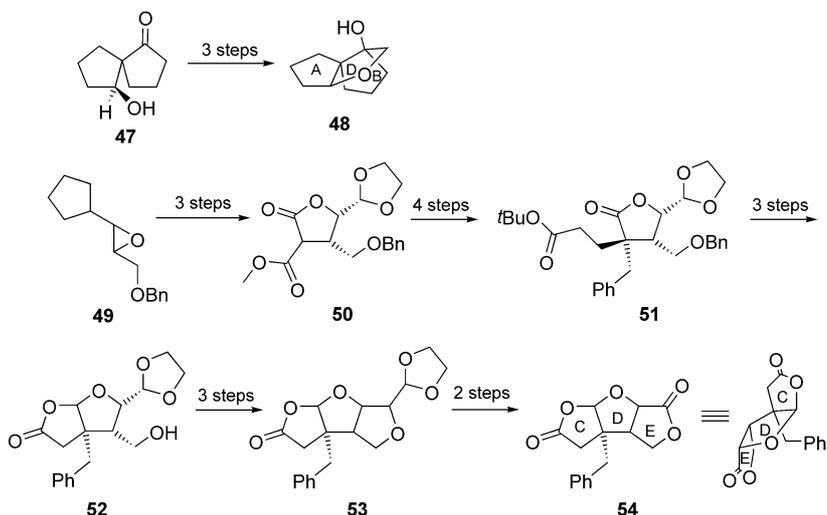
double bonds in **31** were stereospecifically converted into epoxides by treatment with peroxy-3,5-dinitrobenzoic acid to give **32**, which in a sequence of steps involving a selective hydroxylation, acetylation, oxidations, and hydrolysis was converted into trilactone **33** (Scheme 4). Deacetylation and hydrogenolysis of the epoxide of **33** would provide BB (**6**), but this could not be achieved directly. Instead, **33** was deoxygenated, to yield anhydro bilobalide acetate (**34**), which was identical to that previously described.^[76] Dihydroxylation of **34** with osmium tetroxide, followed by deoxygenation of 2-OH gave bilobalide 6-acetate (**35**), which upon exposure to 3 N HCl gave BB (**6**). Thus BB (**6**) was synthesized in 23 steps from **24** (Scheme 4). A stereoselective route to BB (**6**) was reported subsequently in which (+)-menthol was used as a chiral auxiliary in an enone similar to **24**.^[10]

A few years later another total synthesis of BB (**6**) was reported by Crimmins et al., which included two slightly different routes to solve the problem.^[111,112] In the first approach, an intermediate from the Corey synthesis, compound **32** (Scheme 4), was synthesized in 19 steps from 3-furaldehyde by using Sharpless epoxidation, a stereoselective intermolecular [2+2] reaction, and a regioselective Baeyer–Villiger oxidation as key transformations, thus formally completing the total synthesis of BB (**6**). In another approach in which similar transformations were used, the total synthesis of BB (**6**) was accomplished in 17 steps, significantly shortening the synthesis (Scheme 5). In four steps, 3-furaldehyde was converted into aldehyde **36**, which was treated with enol ether **37** in a stereoselective aldol condensation to give enone **38**. Enol pivalate **39** was converted into **40** in an intramolecular, stereoselective [2+2] photocycloaddition, with subsequent hydroxylation to provide **41**. Cleavage of the cyclopentane ring, and formation of acetal **42** was completed in 85% yield, followed by reduction of the esters to give **43**, which was oxidized to furnish cyclobutanone **44**. Treatment of **44**

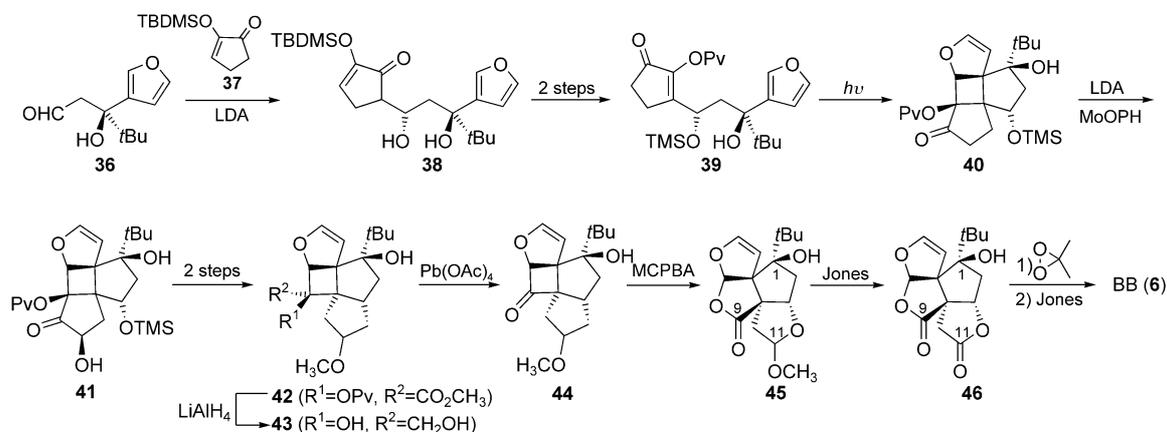
with *m*-chloroperbenzoic acid (MCPBA) in a regioselective Baeyer–Villiger oxidation led to **45**, which provided the basic skeleton of BB (**6**). The total synthesis was completed by treatment with the Jones reagent to give dilactone **46** (Scheme 5), followed by two more oxidations with dimethyldioxirane and the Jones reagent.

4.1.2. Ginkgolides

The first attempt at a total synthesis of ginkgolides was reported by Weinges et al.,^[113] who synthesized compound **48**, a spiro carbocycle ring system fused with a tetrahydrofuran ring system similar to the ABD ring system of ginkgolides, but lacking the *tert*-butyl group (Scheme 6). The synthesis involved the reaction of 6-hydroxyspiro[4.4]nonan-1-one (**47**) in a three-step sequence involving a Grignard reaction, reduction, and cyclization. In 1987, Vilhauer and Andersson synthesized the CDE ring system (**54**, Scheme 6) of the ginkgolide skeleton;^[114] epoxide **49** was converted into lactone **50** by treatment with dimethyl malonate, followed by a Krapcho decarbomethoxylation and two consecutive



Scheme 6. Initial synthetic studies on ginkgolides.



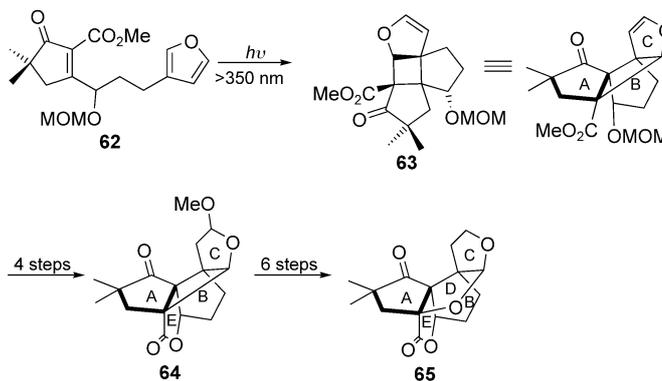
Scheme 5. Total synthesis of BB (**6**) by Crimmins et al. Pv = pivaloyl, TMS = trimethylsilyl, TBDMS = *tert*-butyldimethylsilyl, MCPBA = *meta*-chloroperbenzoic acid.

alkylations with benzyl bromide and *tert*-butyl bromoacetate to give **51**. Deprotection of the acid and subsequent reduction of the lactone, cyclization, and cleavage of the benzyl protecting group gave **52**, which was unstable. Treatment with *p*-TsOH and aqueous acid gave a hemiacetal that was oxidized to **54** with PDC (Scheme 6).^[114] Pattenden and co-workers subsequently reported intramolecular radical cyclization reactions as synthetic entries into the spiro- and linear-fused lactone ring systems found in the ginkgolides.^[115,116] Later, DeLuca and Magnus synthesized a substituted spiro-nonane skeleton, similar to that of the TTLs.^[117]

Corey and co-workers completed the total syntheses of both GA (**1**)^[118] and GB (**2**)^[119] in 1988. The classic total synthesis of GB (**2**) has been extensively reviewed elsewhere^[120–122] and only the key transformations are highlighted herein (Scheme 7). A spirocyclic ring system composed of rings B and C of GB (**2**, Figure 3) was constructed from 2-(2,2-dimethoxyethyl)-cyclopent-2-enone (**55**) by a 1,4-addition of a *tert*-butyl cuprate reagent, followed by a Mukaiyama condensation after treatment with 1,3,5-trioxane and titanium chloride to give **56** (Scheme 7) using the *tert*-butyl moiety as a directing group for the ring closure. A sequence of steps involving a palladium-mediated Sonogashira coupling, an intramolecular ketene–olefin [2+2] cycloaddition, and a Baeyer–Villiger oxidation furnished intermediate **57**, which contains four of the six rings in GB (**2**). For the formation of the tetrahydrofuran moiety, ring D, the reactivity of ring C was modified by introduction of dithiane **58**, and intramolecular etherification provided **59** in five steps. Oxidation of ring A and elimination in ring C furnished **60**, which was selectively oxidized to provide an epoxyketone in ring A. This was followed by an intermolecular aldol condensation and lactonization with concomitant opening of the oxirane to give **61** with all six rings of GB (**2**) in place. The total synthesis was completed by dihydroxylation of the double bond in ring C and oxidation to GB (**2**, Scheme 7). Later, Corey and co-workers suggested an enantioselective route to GB (**2**) by synthesis of a key intermediate,^[123] and various ginkgolide derivatives were also synthesized.^[124,125]

Recently, a novel synthesis of GB (**2**) was reported by Crimmins and co-workers. Their first progress towards the total synthesis of ginkgolides was described in 1989,^[126] with the development of an intramolecular cycloaddition reaction

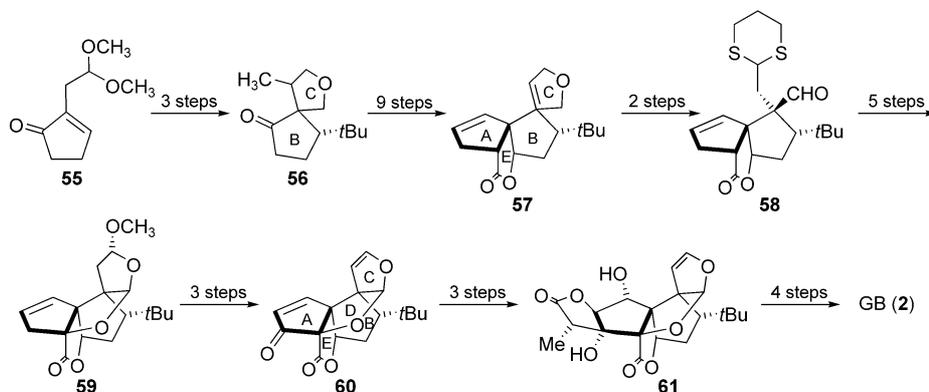
that efficiently generated the multiple-ring skeleton of ginkgolides (Scheme 8). The synthesis started from furan enone **62**, which is very similar to intermediate **39** (Scheme 5)



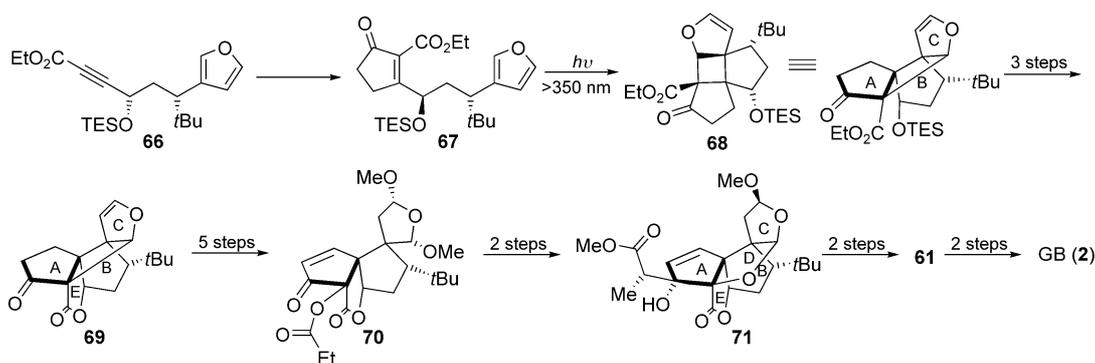
Scheme 8. A photocycloaddition reaction developed and used in synthetic studies on ginkgolides. MOM = methoxymethyl.

encountered in the synthesis of BB (**6**). Furan enone **62** underwent intramolecular photocycloaddition upon irradiation at >350 nm to give tetracyclic **63**, analogous to rings A, B, and C of GB (**2**), as a single diastereomer. Inversion of the MOM-protected alcohol and formation of a bridging lactone gave **64**, and ring expansion of the cyclobutane into a tetrahydrofuran system in six steps gave the desired **65** (Scheme 8), which is comparable to compound **60** (Scheme 7), but lacks crucial functional groups and the *tert*-butyl group.^[126]

In a recently completed total synthesis of GB (**2**, Scheme 9), a photocycloaddition was again used as a key transformation.^[127,128] First, **66** was treated with a zinc–copper homoenolate to give **67**, which was then subjected to irradiation at 366 nm in hexanes, leading to **68** in high yield and excellent diastereoselectivity, establishing two quaternary centers and rings A, B, and C of GB (**2**) (Scheme 9). In three steps, lactone ring F was generated by hydrolysis of the triethylsilyl ether, mesylation of the resulting alcohol, and treatment with pyridinium *p*-toluenesulfonic acid (PPTS) to give **69** in 63% overall yield. X-ray crystallography of a single crystal of **69** confirmed the proposed stereochemistry. Compound **69** was converted into **70** over five steps. Cyclization to form the THF ring by treatment of **70** with LDA, followed by exposure to camphorsulfonic acid (CSA), gave **71**. Treatment of **71** with PPTS led to demethoxylation in ring C. Subsequent Sharpless epoxidation and addition of *p*-TsOH gave a precursor described by Corey, compound **61** (Scheme 7). Two steps were required to complete the total synthesis of GB (**2**) from **61** (Scheme 9).



Scheme 7. Highlights of the total synthesis of GB (**2**) by Corey and co-workers.



Scheme 9. Total synthesis of GB (2) by Crimmins and co-workers. TES = triethylsilyl.

4.2. Synthetic Modification of Parent Compounds

Besides the efforts directed at the total synthesis of ginkgolides and bilobalide from readily available starting materials, numerous synthetic modifications of the parent compounds have been carried out. In particular, several derivatives have been prepared for SAR studies at the PAFR (see Section 5.1.2).

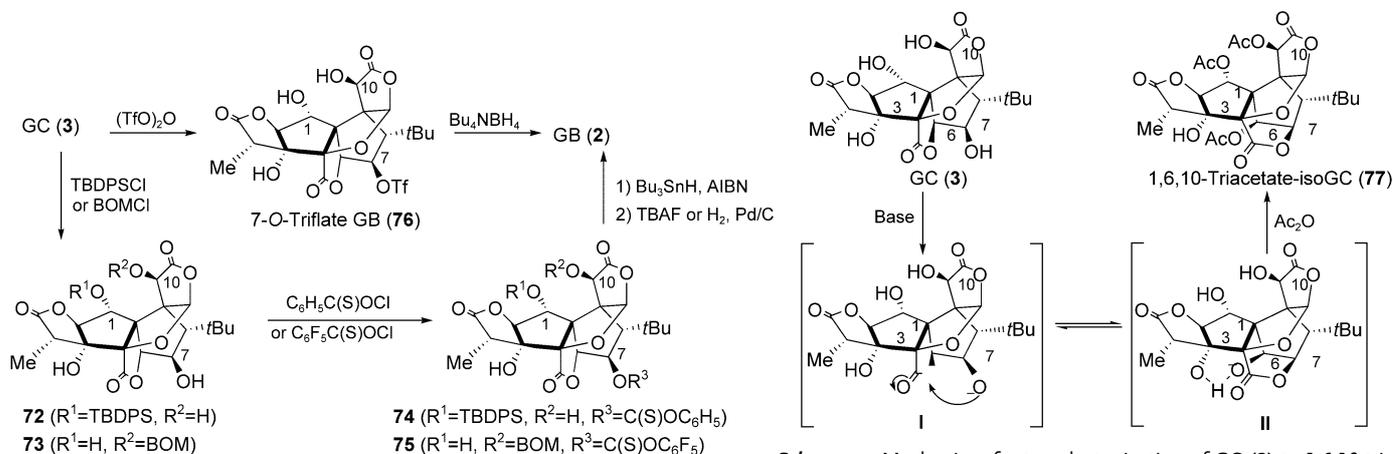
There has been some interest in converting GC (3) into GB (2), the most potent ginkgolide PAFR antagonist, and two approaches have been published. First, Weinges and Schick described a four-step procedure in which the 1-OH group of GC (3) was protected as a *tert*-butyldiphenylsilyl (TBDPS) ether prior to treatment with phenyl chlorothionoformate to give **74** (Scheme 10). This compound was then treated with Bu_3SnH and azobisisobutyronitrile (AIBN) in a Barton–McCombie alcohol deoxygenation, and GB (2) was liberated by removal of the silyl protecting group.^[129] Similarly, Corey et al. protected GC (3) as a benzyloxymethyl (BOM) ether at 10-OH (**73**, Scheme 10), and followed a similar path to form GB (2).^[130] A very convenient, two-step procedure was described in a patent by Teng^[131] in which GC (3) was treated with triflic anhydride to yield exclusively 7-*O*-triflate-GC (**76**), which was reduced with Bu_4NBH_4 to give GB (2) (Scheme 10).

The different reactivities of the 1-, 7-, and 10-OH groups of the ginkgolides are noteworthy: A bulky silyl group reacts

preferentially at 1-OH, whereas all benzyl reagents react at 10-OH and triflic anhydride reacts exclusively at 7-OH. Similarly, it was recently shown that acetylation, which generally takes place at 10-OH, takes place at 7-OH of GC (3) under strongly acidic conditions.^[132] Generally, 1- and 10-OH are the most reactive OH groups owing to the hydrogen bonding between the two and hence the relative ease of formation of a delocalized alkoxy anion.^[130,132] The increased reactivity of 7-OH towards sulfur nucleophiles may be due to the soft-atom nature of sulfur.

During the structural studies of ginkgolides and bilobalide, several analogues were synthesized, some of which were mentioned in Section 2.1. Acetylated ginkgolides and bilobalide^[76,77] were described, as well as the so-called iso-derivatives, which stem from a translocation of ring E in ginkgolides (Figure 3). Recently, an X-ray crystal structure of the iso-derivative 1,6,10-triacetate-isoGC (**77**, Scheme 11) was obtained, and a mechanism for the formation of iso-derivatives that includes opening of ring E, stabilization of the intermediate by hydrogen bonding to 3-OH, and capture by acetic anhydride was suggested (Scheme 11).^[132] A similar translocation was described by Weinges et al. upon acetylation of BB (6) and during the preparation of various acyl derivatives of BB.^[78]

Weinges et al. carried out numerous synthetic studies of ginkgolides and bilobalide.^[133–138] A recent study described an



Scheme 10. Transformations of GC (3) into GB (2). BOM = benzyloxymethyl.

Scheme 11. Mechanism for translocation of GC (2) to 1,6,10-triacetate-isoGC (77).

approach to the preparation of radiolabeled analogues of ginkgolides, in this case [^{14}C]GA, although the actual radioligand was not synthesized.^[139] GA (**1**) was partially degraded as previously described,^[135,136,138] and this was followed by incorporation of the lithium enolate of methyl propionate, which could be [^{14}C]-labeled, and subsequent ring closure to give GA (**1**).^[139]

When ginkgolides are tested in pharmacological assays it is usually necessary to make a concentrated stock solution of the compound in dimethyl sulfoxide (DMSO), owing to their low solubility in water. DMSO may cause problems in certain assay systems,^[140] although such effects are not uniformly observed.^[141] To increase the water solubility of ginkgolides, Weber and Vasella synthesized glycosylated ginkgolide analogues.^[142,143] Glycosidation was carried out by reaction of ginkgolides with a glycosylidene-derived diazirine to give a large number of glycosylated analogues. Intra- and intermolecular bonds of these analogues, as well as the parent ginkgolides, were studied by ^1H NMR spectroscopic analysis.^[144]

Ginkgolides contain three lactones (rings C, E, and F, Figure 3) and hence their structures in solution are highly dependent on the pH value of the media in which they are dissolved. Zekri et al. determined the ionization constants of ginkgolides by ^1H NMR titrations,^[145] which showed that the lactones start to open at around pH 7. At pH 8, the predominant species ($\approx 50\%$) is the form in which only ring F is open; ring E is open to a lesser extent ($\approx 20\%$). An increase in the pH value to 10 opens both rings E and F ($\approx 90\%$). Only at pH 13 is the species with all the lactones open present ($\approx 40\%$), although about 60% is still in the form with only rings E and F open.

5. Pharmacological Effects

In contrast to the wealth of studies on *G. biloba* extracts, far fewer studies have looked at the effect of the individual components of these extracts, in particular the flavonoids and TTLs. Flavonoids and TTLs are believed to be responsible for most of the pharmacological properties of *G. biloba* extracts, and it has been suggested that synergistic effects might be of importance. In any event, studying the effects of the individual components of *G. biloba* extract is essential for providing thorough scientific documentation of potential therapeutic effects of *G. biloba*. A major concern is the bioavailability of these components. It is assumed that the bioavailability of flavonoids is very low,^[21] whereas TTLs, in particular GA (**1**) and GB (**2**), are nearly completely bioavailable.^[146–148] This further underscores the importance of TTLs when looking into effects of *G. biloba* constituents.

5.1. Ginkgolides and the PAF Receptor

In 1985, it was discovered that ginkgolides, particularly GB (**2**), are antagonists of the platelet-activating factor (PAF) receptor.^[149,150] This led to an extensive investigation into the clinical applications of GB (BN 52021) as a PAFR antago-

nist,^[13] but, like all other antagonists of PAFR, GB (**2**) was never registered as a drug, primarily due to lack of efficacy. The clinical studies, however, showed that GB (**2**) was well-tolerated and showed very few, if any, side effects. Today, the most intensively studied activity of TTLs is that of the interaction between GB (**2**) and the PAFR.

5.1.1. The PAF Receptor

The PAFR is a member of the G protein-coupled receptor (GPCR) family and has been identified in a number of cells and tissues, including those in the CNS. In mammalian brains, maximal expression levels were found in the midbrain and hippocampus, with lower levels in the olfactory bulb, frontal cortex, and cerebellum.^[151] PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, **78**, Figure 7), the native phospholipid agonist of the PAFR, is a potent bioregulator that is involved

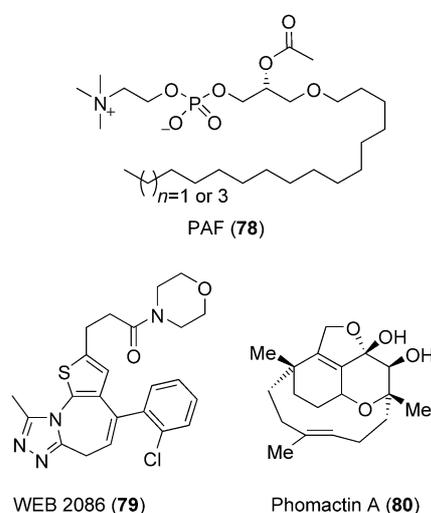


Figure 7. Structures of PAFR ligands.

in the modulation of various CNS and peripheral processes.^[152] PAFR antagonists have been suggested as treatments for various inflammatory diseases, and were pursued by several pharmaceutical companies as antiinflammatory agents. To date, however, no PAFR antagonist has been registered as a drug, but recent evidence indicates that a combination of antibiotics and PAFR antagonists may be a potential treatment of respiratory infections.^[153]

PAF is involved in several events in the CNS, including modulation of long-term potentiation (LTP),^[154–157] increase in intracellular Ca^{2+} ,^[158] and immediate early gene expression.^[159–161] In LTP, PAF is believed to act as a retrograde messenger.^[154] However, PAFR tests on knock-out mice led to different observations: Shimizu and co-workers showed that the PAFR is not required for LTP in the hippocampal CA1 region,^[162] whereas Chen et al. showed that LTP was attenuated in hippocampal dentate gyrus neurons.^[163]

The mechanism by which the PAFR and PAF are involved in the CNS is unclear,^[164] but the PAFR was recently suggested as a target for slowing the progression of neuro-

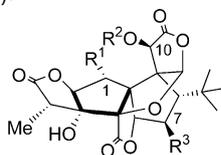
degenerative diseases^[165] and is therefore an important target in unraveling the neuromodulatory effects of TTLs.^[166,167]

5.1.2. SAR Studies of Ginkgolides

A remarkable feature of PAFR antagonists is their structural diversity, ranging from WEB2086 (**79**) and phloctactin A (**80**) (Figure 7) to ginkgolides, all structurally very different from PAF, but still competitive antagonists. Until recently, SAR studies of ginkgolides on the PAFR focused on derivatives of ginkgolide B (GB, **2**), the most potent antagonist of the PAFR, and in all these cases, the derivatives were evaluated for their ability to prevent PAF-induced aggregation of blood platelets (in rabbits).

In the initial description of the ability of ginkgolides to inhibit the PAFR, it was shown that GB (**2**) was the most potent TTL ($IC_{50} = 0.25 \mu\text{M}$), GA (**1**) was slightly less potent, and GC (**3**) was a very weak antagonist (Table 1).^[149] A few years later, methoxy and ethoxy analogues of GA (**1**), GB (**2**), and GC (**3**) were prepared in which the alkyl groups were introduced at C1 or C10 by reaction with diazoalkane to yield mixtures of 1- and 10-substituted analogues, which were separated by column chromatography.^[168] Interestingly, 1- and 10-methoxy analogues of GB (**2**) were equipotent to GB (**2**), whereas the corresponding ethoxy analogues were less potent. The 10-substituted analogues of GA (**1**) were significantly less potent than GA (**1**), whereas methyl analogues of GC were more potent, and ethyl analogues were equipotent to GC (**3**) (Table 1).

Table 1: Pharmacological activity of methoxy and ethoxy analogues of GA (**1**), GB (**2**), and GC (**3**).



Compounds	R ¹	R ²	R ³	IC ₅₀ [μM]
GA (1)	H	H	H	0.74
GB (2)	OH	H	H	0.25
GC (3)	OH	H	OH	7.1
10-Me-GA	H	CH ₃	H	13
10-Et-GA	H	CH ₃ CH ₂	H	62
1-Me-GB	OCH ₃	H	H	0.66
10-Me-GB	OH	CH ₃	H	0.29
1-Et-GB	OCH ₃ CH ₂	H	H	1.1
10-Et-GB	OH	CH ₃ CH ₂	H	7.2
1-Me-GC	OCH ₃	H	OH	4.2
10-Me-GC	OH	CH ₃	OH	3.0
1-Et-GC	OCH ₃ CH ₂	H	OH	8.5
10-Et-GC	OH	CH ₃ CH ₂	OH	9.3

Corey et al. investigated various intermediates in the total syntheses of GA (**1**) and^[118] GB (**2**)^[119] and found that the lactone F (Figure 3) was not essential for activity and could be replaced by other lipophilic groups,^[169] whereas the unique *tert*-butyl group was critical for activity.^[125] Vilhauer and Anderson synthesized **54** with the CDE ring system of

ginkgolides (Section 4.1.2, Scheme 6) and investigated its ability to antagonize the PAFR.^[114] This moiety of the ginkgolide structure was found to be ineffective as a PAFR antagonist, which provided important information about structural requirements for PAFR inhibition by the ginkgolides.

The most comprehensive SAR study on ginkgolides and PAFR was performed by Park et al., who synthesized more than 200 derivatives of GB (**2**), with particular focus on aromatic substituents at 10-OH.^[170] These derivatives were generally synthesized by treatment of GB (**2**) with a base and a benzyl halide derivative to provide, in most cases, selective derivatization at 10-OH. Most of the 10-*O*-benzylated derivatives were more potent than GB (**2**) ($IC_{50} = 0.258 \mu\text{M}$), for example, 10-(3,5-dimethyl-2-pyridinyl)-methoxy-GB ($IC_{50} = 0.0245 \mu\text{M}$) was tenfold more potent than GB (**2**). The same group also investigated elimination products of GB as well as derivatives bridged between 1- and 10-OH, but all these analogues were much less potent than GB (**2**).^[171] Hu et al. used a slightly modified procedure to prepare GB (**2**) derivatives, many of which were identical to those synthesized by Park et al. Not surprisingly, they also obtained benzylated GB derivatives that were more potent than GB (**2**).^[172,173] Later, Hu et al. prepared various degradation and elimination products of GA (**1**) and GB (**2**) as well as amide derivatives that lack rings C and D (Figure 3), but in all cases decreased PAFR antagonism was observed.^[174,175]

One goal of SAR studies is to put forward a pharmacophore model that can elucidate the activities of synthesized derivatives as well as predict the activity of novel derivatives. A three-dimensional quantitative SAR (3D-QSAR) study^[176] was attempted for ginkgolides and the PAFR, using comparative molecular field analysis (CoMFA) and 25 ginkgolide analogues, mainly those synthesized by Corey et al.^[118,119,169] In agreement with the SAR studies recently described, this pharmacophore model predicted that substituents at 10-OH of GB would improve activity. Moreover, a density functional theory (DFT) calculation of the geometry of GB (**2**) confirmed the X-ray crystal structure. The same calculation was also used to predict certain IR stretching bands.^[177]

Clarification of the interactions between ginkgolides and PAFR at the molecular level can be carried out with photolabeling techniques. Therefore, we recently prepared photoactivatable derivatives of GB (**2**) and GC (**3**)^[141] and generated highly potent analogues with 4-(bromomethyl)-benzophenone (**81**), trifluoromethyldiazirine (**82**), and tetrafluorophenyl azide (**83**) groups at the 10-OH position of GB (Figure 8) as the most active ($K_i = 90\text{--}150 \text{ nM}$). These derivatives are promising tools for characterizing the PAFR–ginkgolide interaction. This study also provided the first evaluation of the interaction of ginkgolides with a cloned PAFR by means of a radioligand-binding assay. In another recent study, the effect of acetate derivatives of ginkgolides was investigated which showed that acetylation generally decreases the antagonistic effects at the PAFR,^[132] thereby suggesting that GB diazoacetates would likely not be useful for photolabeling studies. Most recently, a study of the effect of substitutions at the C7 of the ginkgolides showed that in contrast to previous reports, very potent ginkgolide deriva-

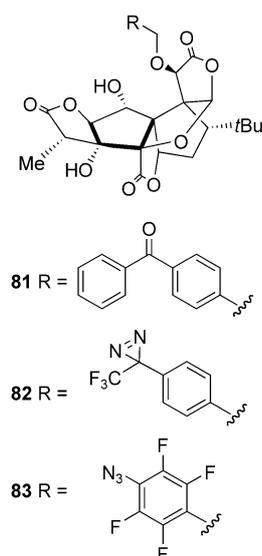


Figure 8. Photoactivatable analogues of ginkgolides.

tives could be prepared by introducing chlorine, for example, 7-chloro-GB ($K_i = 110$ nm) is eightfold more potent than GB (**2**).^[178]

The large number of ginkgolide derivatives that have been prepared and tested for their ability to antagonize the PAFR have led to a clearer understanding of the required structural features (summarized in Figure 9). Further investigation is required to determine the molecular structural interaction of TTLs with the PAFR as well as the potential physiological effects in the CNS functions.

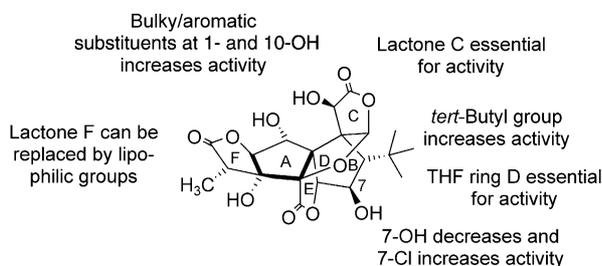


Figure 9. Summary of the SAR studies on ginkgolides and the PAFR.

5.2. Ginkgolides and Glycine Receptors

Until very recently, the only specific target for ginkgolides has been the PAFR, whose importance to CNS function is not clear. Therefore, the recent finding that GB (**2**) is a potent and selective antagonist of glycine receptors (GlyRs)^[179–181] provided the first indications as to how ginkgolides may exert their effect in the CNS. The GlyRs are found primarily in the spinal cord and brain stem, but also in higher brain regions such as the hippocampus. They are, together with γ -aminobutyric acid receptors (GABA_ARs), the main inhibitory receptors in the CNS.^[182, 183] Both GlyRs and GABA_ARs are

ligand-gated ion channels that, together with nicotinic acetylcholine (nACh) and serotonin (5-HT₃) receptors, constitute a superfamily of membrane receptors that mediate fast chemical synaptic transmission in the CNS.^[184] GlyRs share several structural similarities with these receptors, including a pentameric arrangement of subunits, each composed of four transmembrane domains (M1–M4) and an extracellular N-terminal 15-residue cysteine-loop motif.^[185] Recently, there has been a renewed interest in ligands for GlyRs, as modulators of GlyR function could be used as muscle relaxants, as well as sedative and analgesic agents.^[186]

Electrophysiological studies showed that GB (**2**) antagonizes glycine receptors in neocortical slices^[181] and hippocampal cells,^[179] and that the inhibition is noncompetitive, use-dependent, and probably voltage-dependent, thus suggesting that GB (**2**) binds to the central pore of the ion channel. It was also shown that GC (**3**) and GM (**5**) were almost equipotent to GB (**2**), whereas GA (**1**) and GJ (**4**) were significantly less potent,^[180, 181] suggesting an important function of the 1-OH group present in GB (**2**), GC (**3**), and GM (**5**), but absent in GA (**1**) and GJ (**4**). This assumption was corroborated by molecular modeling studies which showed a striking structural similarity between picrotoxinin, an antagonist of both GABA_ARs and GlyRs, and ginkgolides.^[181] Thus, ginkgolides are highly useful pharmacological tools for studying the function and properties of GlyRs. However, the antagonism of inhibitory receptors might have serious implications for people taking *G. biloba* extract (see Section 5.5).

5.3. Ginkgolides and the Peripheral Benzodiazepine Receptor (PBR)

Benzodiazepines are used clinically as anticonvulsants and anxiolytics, effects that are mediated through binding to a specific benzodiazepine site on GABA_ARs located in the CNS. Benzodiazepines, however, also bind to receptors located mainly in peripheral tissues and glial cells in the brain. These receptors are called peripheral benzodiazepine receptors (PBRs)^[187, 188] and are typically located on the outer membranes of mitochondria. The function of PBRs is not entirely clear, but they have been suggested to be involved in steroidogenesis, cell proliferation, and stress and anxiety disorders. The latter theory is supported by an increase in the number of PBRs in specific brain regions in neurodegenerative disorders and after brain damage.^[187]

Several studies have shown that ginkgolides, particularly GA (**1**) and GB (**2**), can modulate PBRs. Initially it was shown that the ligand-binding capacity of PBRs decreased with decreasing protein and mRNA expression.^[189] This led the authors to suggest that the neuroprotective effects of GA (**1**) and GB (**2**) could be explained by their effect on glucocorticoid biosynthesis.^[189, 190] Recent studies have shown that the primary action of GB (**2**) is the inhibition of PBR expression,^[191] which is mediated through binding to a transcription factor, and it has been suggested that GB (**2**) regulates excess glucocorticoid formation through PBR-controlled steroidogenesis.^[192, 193]

5.4. Various Effects of the Ginkgolides

The interactions of ginkgolides with the PAFRs, GlyRs, and PBRs described above are the best-characterized interactions of ginkgolides with targets found in the CNS. Varying effects of ginkgolides were observed in numerous assays with different tissues and conditions. None of these studies provided a clear-cut target for ginkgolides, but instead introduced a vast number of different pharmacological effects that may or may not be related to the targets described.

Several studies have indicated that ginkgolides protect against various CNS incidents, such as ischemia and cerebrovascular and traumatic brain injury, as well as inflammation.^[18] GB (2) is believed to interfere with postischemic production of free oxygen radicals,^[194] and it has been shown that GA (1) and GB (2) decreases glutamate-induced damage of neuronal^[195] and hippocampal cells.^[196] One study credited GB (2) with protection against the decrease in hippocampal Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) activity after cerebral ischemia.^[197] This is interesting as CaMKII is believed to be involved in LTP, and this could provide an explanation for the neuromodulatory effects of ginkgolides.

GA (1) and GB (2) were recently shown to reduce the amount of potentially cytotoxic nitric oxide produced by inducible nitric oxide synthase (iNOS),^[198] an effect also observed for BB (6). Similarly, ginkgolides were shown to have cardioprotective effects, with GA (1) being the most effective.^[194,199] To prove that this effect was not related to PAFR antagonism, a GC (3) derivative with a tolyl group at 7-OH was synthesized. This derivative showed improved cardioprotective activity relative to GB (2) and GC (3), while having no effect at the PAFR at 120 μM.^[200] Finally, the few clinical studies performed with GB (2) showed its effects on peripheral events, such as efficacy against Gram-negative-induced septic shock^[201] and post-transplant renal failure,^[202] which is most likely to be due to the inhibition of the PAFR.

5.5. Bilobalide

Bilobalide (BB, 6) is the predominant TTL found in the standardized *G. biloba* extract EGb 761. No specific target has been identified for BB (6) and hence no SAR studies have been carried out. As BB (6) is far more labile than the ginkgolides, its chemistry is somewhat limited. To date, only various acetylations of BB (6) have been carried out successfully.^[75–78] Although no specific target has been established and pursued, a wealth of pharmacological evidence indicates that BB (6) might be a very important compound when looking at the neuromodulatory properties of *G. biloba* constituents.^[203]

Several studies have indicated that BB (6) affects the major neurotransmitters in the brain, namely glutamate and γ -aminobutyric acid (GABA). It was demonstrated that BB (6) exhibits anticonvulsant activity against convulsions induced by isoniazid, pentylenetetrazole, and 4-*O*-methylpyridoxine.^[204,205] Later it was shown that this effect is most likely mediated by increased GABA and glutamic acid decarbox-

ylase (GAD) levels in various areas of the mouse brain.^[206,207] Moreover, in rat hippocampal brain slices, BB (6) induced an enhancement of excitability and attenuated the inhibitory action of muscimol, a potent GABA_AR agonist, thus indicating that BB (6) reduces GABA-related transmission,^[208] a finding that apparently seems to contradict previous findings by the same group.

Recently, it was unequivocally shown that BB (6) is a GABA_AR antagonist. In neocortical rat brain slices, BB (6) was a weak (IC₅₀ = 46 μM) noncompetitive antagonist,^[181] whereas it was more potent at recombinant $\alpha_1\beta_1\gamma_{2L}$ GABA_ARs and showed some degree of competitive antagonism.^[209] Since antagonists of GABA_ARs are known convulsants, this could be a potential risk when taking *G. biloba* extract. This risk was further substantiated by a study of two epileptic patients, who had an increased frequency of seizures when taking *G. biloba* extract; this increase was reversed when the patients stopped taking the extract.^[210] These results indicate that people with a lower seizure threshold, such as epileptic patients, should be cautious when taking *G. biloba* extract.

In rat cortical brain slices under hypoxic/hypoglycemic conditions, bilobalide significantly reduced glutamate release, suggesting that the neuroprotective effects of BB (6) might be mediated by reduced glutamate efflux and, thereby, excitotoxicity.^[211] It was also shown that BB (6) could reduce potassium- and veratridine-induced release of excitatory amino acids such as glutamate and aspartate, and block the effect of a GABA uptake inhibitor, NO-711.^[212] Weichel et al. found that BB (6) inhibited *N*-methyl-D-aspartate (NMDA)-induced phospholipid breakdown in rat hippocampus and suggested an effect downstream of the NMDA receptor.^[213] However, it was recently found that BB (6) does not affect NMDA-induced depolarizations, strongly suggesting that it had no effect on the NMDA receptor.^[212] Potential medicinal applications of BB (6) have been described in patents, including the use of BB (6) for the protection of neurons from ischemia,^[214] as an anticonvulsant,^[215] and for the treatment of tension and anxiety.^[216]

Two other targets were also described for BB (6): phospholipase A₂ (PLA₂) and mitochondrial respiration. BB (6) inhibits brain PLA₂ activity and hypoxia-induced increase in choline influx^[213,217,218] and also protects against hypoxia-induced PLA₂ activation.^[219,220] Another study indicated a neuroprotective effect by reduction of the brain infarct area following ischemia.^[221] A number of studies have revealed that BB (6) is involved in mitochondrial respiration, especially under ischemic conditions.^[222] BB (6) was also shown to increase glucose transport under normoxic but not hypoxic conditions, increase respiratory control of mitochondria, and inhibit ATP consumption as a result of increased respiratory efficiency.^[223]

Finally, there is an indication that the effect of EGb 761 on β -amyloid aggregation and potential protection against AD might be, at least in part, mediated by BB (6).^[39] However, more studies are required to confirm this.

6. Summary and Outlook

The bilobalide and ginkgolide structures have been known for about 35 years, and since then a vast number of chemical and biological studies have been carried out. The total syntheses of these complex natural products rank among the major accomplishments in natural products synthesis. The first significant biological activity of ginkgolides was discovered in 1985, when it was shown that they are potent antagonists of the PAF receptor, thus providing a potential treatment for PAF-related maladies. Ginkgolides and bilobalide were characterized in a wealth of different pharmacological assays, particularly the recent finding of their ability to antagonize inhibitory receptors in the brain.

Within the last couple of years the literature on *G. biloba* in general and terpene trilactones in particular has expanded rapidly. As new biological targets are discovered and already-existing targets are more thoroughly explored, a better understanding of the ligand–receptor interaction at a molecular structural level will provide new insight into the actions of the unique constituents of the oldest living plant.

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