Preparative Isolation and Separation Procedure for Ginkgolides A, B, C, and J and Bilobalide

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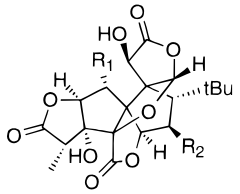
A simple preparative method for the isolation and purification of ginkgolides A, B, C, and J and bilobalide (ginkgo terpene trilactones) was developed. As starting material, a commercially available leaf extract from *Ginkgo biloba* containing ≥ 6% ginkgo terpene trilactones was used. After a partition step with EtOAc, the enriched intermediate extract was separated into the individual terpenes by medium-pressure liquid chromatography on silica impregnated with 6.5% NaOAc with a gradient from petroleum ether−EtOAc to EtOAc−MeOH. After recrystallization from H_2O−MeOH, all five terpenes could be isolated in high purity. After a selective extraction with H_2O, leaves could also be used as a starting material.

Ginkgolides A, B, C, and J (**1**−**4**) and bilobalide (**5**) are unique, highly oxidized terpene trilactones from the leaves of *Ginkgo biloba* L. (Ginkgoaceae) (maidenhair tree). Ginkgolides are potent and selective platelet-activating-Factor antagonists and are considered to be at least partially responsible for the medicinal properties of ginkgo extracts.[1] The structures of the ginkgolides A, B, and C [further abbreviated as G-A (**1**), G-B (**2**), G-C (**3**)] were independently elucidated by two Japanese groups in the 1960s.[2,3] Ginkgolide J (G-J) (**4**) and the related sesquiterpene bilobalide (**5**) have been elucidated by Weinges *et al.*[4−6]

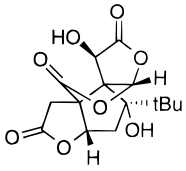


1: Ginkgolide A $R_1 = R_2 = H$

2: Ginkgolide B $R_1 = OH, R_2 = H$

3: Ginkgolide C $R_1 = R_2 = OH$

4: Ginkgolide J $R_1 = H, R_2 = OH$



5: Bilobalide

Several different procedures have been published for the isolation and separation of these terpenes; however, due their relatively low abundance in leaves (0.001−0.8%), the presence of many other secondary metabolites in higher concentrations, and the difficult separation of the pairs G-A/G-B and G-J/G-C, their purification is tedious. Until now, these pairs have been separated only by a 10−15-step fractional crystallization, by repeated column chromatography,[7] or by reversed-phase HPLC.[8−10] Because of this difficulty, Corey has even suggested to derivatize a mixture of G-A and G-B, to separate the derivatives (which is simple), and then to convert them back to the pure ginkgolides.[11] Because of the potential usefulness of pure G-B or a mixture of ginkgolides as novel drugs against asthma and shock as well as for quality control purposes, there is a high demand for the pure compounds. This demand, the lack of commercial availability of larger quantities in 95+% purity, and the difficulties connected with existing purification methods have prompted us to search for a more efficient isolation and separation method, which is presented below.

The most important aim of our investigations was finding a simple, column chromatographic step for the preparative separation of ginkgolides A/B and C/J. As a starting point, the published separation of bilobalide and all four ginkgolides by means of TLC on Si gel impregnated with NaOAc was used.[12] For bilobalide, G-A, G-B, G-J, and G-C, R_f values of 0.58, 0.41, 025, 0.12, and 0.06, respectively, were reported. The exact role of the NaOAc is not known. However, experiments with other salts have shown that both the sodium and the acetate ion influence the separation (van Beek and Lelyveld, unpublished results). Most likely the salt interferes with the strong internal hydrogen bonding between the hydroxyls at C-1 and C-10 in G-B and G-C. Because of this hydrogen bonding, G-B and G-C are approximately equally polar as G-A and G-J, respectively, and have similar chromatographic behavior under both normal and reversed-phase conditions. Therefore, this TLC procedure was replaced by a medium pressure liquid chromatographic (MPLC) procedure. For this purpose, TLC-quality Si gel was impregnated with various percentages of NaOAc, and trial MPLC separations of ginkgolides were carried out. Solvent mixtures of EtOAc and petroleum ether 40°/60° were used. Analysis of collected fractions was carried out by means of TLC,[12] HPLC,[13] or NMR.[14]

The results were promising, and further optimization showed that best results were obtained with 6.5% (g/g)

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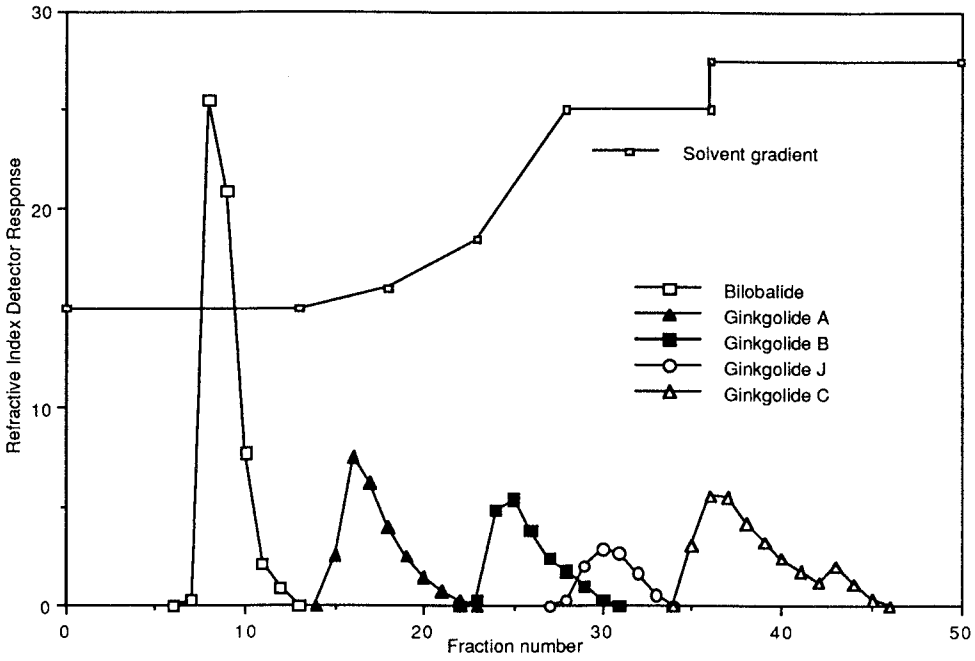

Figure 1. Result of a preparative separation of 500 mg enriched ginkgo terpene trilactone fraction by means of MPLC (2 cm i.d.). Stationary phase: silica impregnated with 6.5% NaOAc. Gradient from petroleum ether−EtOAc 30:70, via petroleum ether−EtOAc 27:73, petroleum ether−EtOAc 20:80, 100% EtOAc to EtOAc−MeOH 98:2.

NaOAc impregnation. Because of the large difference in polarity between bilobalide and G-C, a gradient solvent system was necessary to finish the separation within a reasonable amount of time. The initial solvent was petroleum ether 40°/60°: EtOAc = 30:70. In three steps, the percentage of EtOAc was increased to 100%. Finally, to speed up the elution of the most polar G-C, 2% MeOH was simply added to the EtOAc flask. This percentage of MeOH did not displace any NaOAc acetate from the column, and the column could be regenerated by washing with several column volumes of the starting solvent. A chromatogram of an average separation is depicted in Figure 1. There is a baseline separation of bilobalide, G-A, and G-B and a base-line separation of G-J and G-C. The only ginkgolides that were usually not fully separated were G-B and G-J. Their separation however, is simple by means of either low-resolution normal (silica without impregnation) or reversed-phase (C18) flash chromatography. In several cases, even spontaneous crystallization of G-B occurred when, after evaporation, mixed G-B/G-J fractions were redissolved in MeOH−H_2O mixtures.

Having achieved this first goal, the next, less difficult, step was the development of a simple preparative extraction−clean-up step that would yield an enriched mixture of ginkgo terpene trilactones. Two different starting materials can be used for a preparative isolation: (a) dried ginkgo leaves and (b) a commercially available standardized ginkgo extract containing 24% flavonoid glycosides and 6% terpene trilactones. Both sources have advantages and disadvantages. Dried ginkgo leaves contain highly variable amounts of ginkgo terpene trilactones (0.001%−0.8%), which makes a quantitative determination prior to the extraction process a prerequisite.[13,15,16] Preferably, leaves should contain more than 0.2% total terpene trilactones. For instance, for obtaining 1 g of the pharmacologically most active G-B, around 5 kg of 0.2% leaves must be extracted. Working with such relatively large amounts of plant material may pose a problem for some laboratories. The same amount of G-B can be obtained from only 100 g of the commercially available standardized extract. Filtration problems are about equal for both sources. Surprisingly, with the developed clean-up procedure (see below), the final extract for the MPLC separation obtained from leaves was not any more contaminated than the corresponding extract from the commercial standardized source. Apparently, some troublesome impurities occur in the standardized extract, which have been enriched together with the terpene trilactones. These impurities are probably not extracted from dried leaves with the method described below.

Although many isolation procedures for ginkgolides start with an initial extraction with Me_2CO, Me_2CO−H_2O, or alcohol−H_2O, often followed by partitioning the extract with hexane to remove apolar impurities such as chlorophyll, we feel that an initial extraction with H_2O alone or H_2O with 10% MeOH is a more selective and simple procedure. This procedure was initially put forward by Okabe *et al.*[3] and has later become part of a validated quantitative extraction procedure.[13] Ginkgolides are quite soluble in boiling H_2O without any decomposition taking place. After filtration and addition of 10% (g/v) salt, the aqueous extract is extracted with EtOAc giving a quantitative extraction of all ginkgo terpene trilactones including the polar G-C. Ethyl acetate was chosen because it is an excellent solvent for all compounds of interest, has a relative low cost, exhibits low toxicity, and has good biodegradability. It should not be replaced by more apolar solvents like Et_2O because G-C is poorly soluble in such solvents. After drying with anhydrous Na_2SO_4 and evaporation *in vacuo*, a brown, sticky mass resulted that contained approximately 25% total terpene trilactones.

An additional enrichment step was carried out immediately prior to the injection on the MPLC column. A quantity of the EtOAc extract was dissolved with

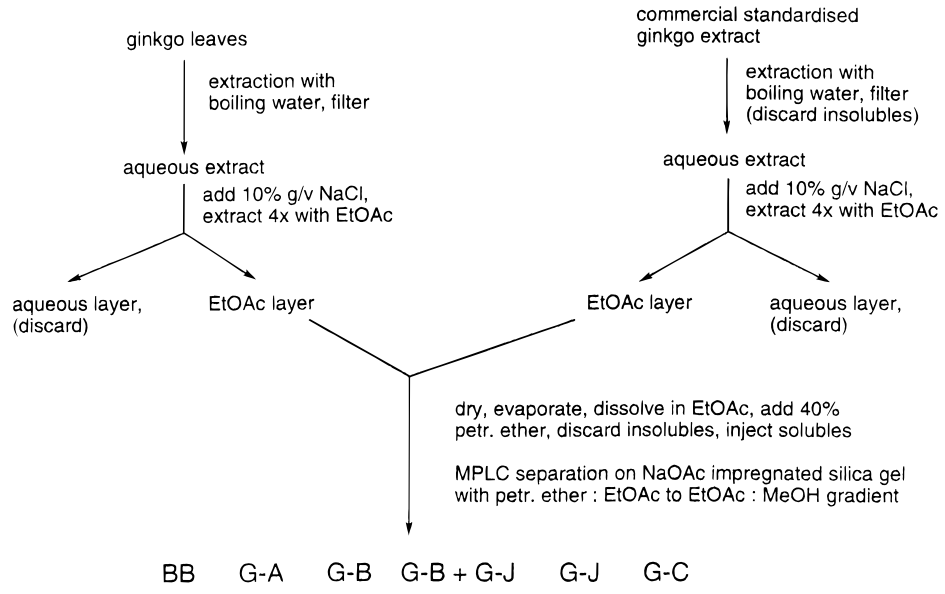

Figure 2. Schematic representation of the preparative isolation and purification procedure for bilobalide and ginkgolides A, B, C, and J.

sonification in a tenfold (v/g) volume of EtOAc, 43% of petroleum ether 40°/60° was added, and the resulting precipitate was filtered and discarded. The resulting clear light brown solution was directly injected. The final solvent composition of the sample was identical to that of the starting MPLC solvent (petroleum ether−EtOAc 30:70). In this last precipitation step some more polar impurities were removed. The total clean-up and separation procedure is presented schematically in Figure 2. All fractions that were discarded contained minimal quantities of ginkgo terpene trilactones according to TLC and HPLC.

For the crucial MPLC separation step, initially 2- and later 4-cm internal diameter axial compression columns were used. On these columns, 0.5 g and 2 g of the twice-enriched extract (corresponding to ca. 0.18 g and 0.7 g total ginkgo terpene trilactones), respectively, could be applied, giving typically a chromatogram similar to that illustrated in Figure 1. In some cases a base-line separation of G-B and G-J was observed. After screening of the fractions with TLC, fractions containing the same compound were pooled and evaporated. At this stage, the five ginkgo terpene trilactones were not yet totally pure. Further purification to white powders with a purity of 98+% was achieved by a combination of crystallization, treatment with activated charcoal or MPLC on 40-μm C18 material (G-B and G-J). Final purity was assessed by TLC, reversed-phase HPLC, and qualitative and quantitative NMR.

With the above procedure, it is now possible to obtain baseline separation of G-A and G-B and G-J and G-C on a 300-mg scale and to obtain within a reasonable amount of time sufficient amounts for multiple calibrations. All of this is possible without resorting to very expensive preparative reversed-phase HPLC. This is of particular importance for the quality control of the ginkgo terpene trilactone content in ginkgo leaves, standardized extracts, and phytopharmaceuticals as these substances are now either not for sale at all (bilobalide, G-C, G-J) or available only in small quantities of limited purity at high prices (G-A, G-B).

Experimental Section

General Experimental Procedures. Chemicals were of analytical grade. Solvents for HPLC were of HPLC grade. Solvents for MPLC were distilled before use. NMR spectra were recorded on a Bruker AC-E 200 NMR spectrometer equipped with an autosampler.[14] TLC and HPLC were carried out as described earlier.[12,13]

Preparation of Impregnated Stationary Phase. First a smooth suspension of 467.5 g silica (Si PF_{254} for preparative TLC, Merck 7747) and 32.5 g NaOAc in MeOH was prepared. This suspension was transferred to a specially made roundbottom flask with internal notches. After removing the MeOH with a rotary evaporator under constant stirring, the impregnated silica was transferred to an open porcelain bowl and dried overnight at 105 °C in an oven with forced-air circulation. After cooling the silica was ready for use. The silica was stored in hermetically sealed tin drums.

Enrichment of Standardized Extract. Approximately 75 g of standardized ginkgo extract was transferred in small portions into 1 L boiling H_2O. After 10 min of stirring, the still-boiling solution was filtered through a Büchner funnel. The remaining insolubles were extracted a second time in a similar fashion with 200 mL H_2O. After cooling and addition of 10% (g/v) NaCl, the combined filtrates were extracted five times with 300 mL EtOAc each. After overnight drying with anhydrous Na_2SO_4, the combined organic layers were evaporated *in vacuo*, giving 21.6 g enriched extract. A further precipitation step just prior to the MPLC separation was used to remove more polar impurities. Of the enriched extract, 3 g was dissolved in 28 mL EtOAc after which 12 mL petroleum ether 40°/60° was added. After centrifugation the clear supernatant was pipetted off and filtered over a 0.45-μm membrane filter (RC55). This solution, containing 1.9 g of solid material, was injected on the MPLC.

Extraction and Purification of Leaves. Approximately 250 g dry ginkgo leaves were extracted for 15

min with boiling distilled H_2O. After centrifugation and decantation, the leaves were once more extracted in a similar fashion with 650 mL H_2O. After centrifugation the filtrates were combined (1950 mL) and 195 g NaCl was added. The aqueous solution was then extracted four times with 500 mL EtOAc each. Any emulsion formed was broken by centrifugation. The combined organic layers were dried overnight with anhydrous Na_2SO_4. After removal of the solvent *in vacuo*, 3.6 g amorphous powder remained. This crude extract could be further enriched in ginkgo terpene trilactones by the same precipitation step as used for the standardized extract (see above).

MPLC. MPLC separations were carried out on a Jobin Yvon 50 × 2 or 50 × 4 cm LC column with axial compression. The columns were filled with a slurry of the impregnated silica (see above) in the initial eluent (petroleum ether 40°/60°−EtOAc 3:7), closed, and pressurized (ca. 10 atm). Until they were discarded, the columns remained under continuous pressure to avoid the formation of voids. The sample was injected by means of an HPLC injection valve with a 10-mL loop made of Teflon tubing. If more than 10 mL had to be injected (in case of the 4-cm i.d. column), after the first injection the pump was run for 1 min at a flow of 10 mL/min. The loop was then refilled with sample, after which the pump was restarted. This could be repeated until the desired volume had been injected. The flow was 4 and 16 mL/min for the 2- and 4-cm i.d. columns, respectively. The eluent was delivered to the column by means of two Gilson 303 HPLC pumps. Eluent A: petroleum ether 40°/60°−EtOAc 30:70; eluent B: EtOAc = 100. During the separation a gradient was run: start until fraction 13: 100% A; fraction 13 to fraction 18: 100% A to 90% A; fraction 18 to fraction 23: 90% A to 65% A; fraction 23 to fraction 28: 65% A to 0% A; fraction 28 to fraction 37: 100% B; fraction 37 to fraction 46: EtOAc−MeOH 98:2. The gradient was controlled by Gilson gradient software on an Apple 2E computer. Fractions (24 mL and 96 mL for 2- and 4-cm columns, respectively) were collected with a Gilson 202 fraction collector. Fractions were screened for ginkgolide content by TLC, NMR, or HPLC. The column could be regenerated for a subsequent separation without loss of chromatographic resolution by flushing with 4 column volumes (ca. 1 L) of solvent A.

Final Purification. Final purification of pooled MPLC fractions containing only one terpene trilactone was carried out by recrystallization. G−J, G−C, G−A, and G−B were recrystallized three to four times from MeOH−H_2O 1:4, MeOH−H_2O 1:3, MeOH−H_2O 35:65, and MeOH−H_2O 4:6, respectively. Bilobalide was recrystallized from MeOH−0.1% TFA in H_2O 15:85. Compounds were dissolved in a sufficient quantity of the appropriate solvent mixture at 45 °C and then left in the refrigerator overnight. All ginkgolides were then obtained as white crystals; bilobalide, however, remained off-white. To further purify bilobalide, the off-white crystals were dissolved in EtOAc, stirred with some activated charcoal (Norit), filtered over 2 cm Hyflo and evaporated *in vacuo*. A clear white amorphous powder resulted. MPLC fractions containing both G-B and G-J could be easily separated into the individual terpene trilactones by means of axial compression MPLC (see above) on 40-μm C18 material (Baker) with MeOH−H_2O 35:65 as solvent. They were then further purified by recrystallization as described above.

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