Efficient Extraction of Ginkgolides and Bilobalide from Ginkgo biloba Leaves

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An efficient and rapid protocol has been developed for extracting ginkgolides and bilobalide (terpene trilactones) from Ginkgo biloba leaves. The procedure takes advantage of the extraordinary stability of the terpene trilactone structure to a variety of chemical treatments, especially oxidation, despite the presence of multiple oxygen functions. The protocol involves boiling the aqueous extract of leaves with dilute hydrogen peroxide, extraction with ethyl acetate, washing with basic solutions, and charcoal filtration to yield an off-white powder, terpene trilactone content 60–70%. It is likely that the hydrogen peroxide treatment degrades the undesired leaf constituents that lead to intense emulsification during extractions. Further reversed-phase chromatography of the extracts with polymeric resins removes the undesirable ginkgolic acids to amounts less than 10 ppm. The extracts are suited for pure terpene trilactone preparation, enrichment of terpene trilactone content in nutraceuticals, and preparations of low-flavonoid/high-terpene trilactone controls in medicinal studies. The four ginkgolides (ginkgolides A, B, C, J) and bilobalide isolated from the extract were identical in all respects with authentic specimens.

The Asian tree Ginkgo biloba, mentioned in the Chinese Materia Medica 5000 years ago,1 has generated scientific interest for its reputed value in treatment of memory-related afflications. Recent research has focused on the medicinal value of the leaves;2 approximately 8000 tons of leaves are harvested globally to address the commercial demand for G. biloba products.3 EGB 761,4 a standardized extract of the leaves, contains 5–7% ginkgolides5,6 and bilobalide (BB)7 (terpene trilactones) (Figure 1) along with 22–24% flavonoids and less than 5 ppm ginkgolic acids (also known as anacardic acids).4 This limit was imposed because alkylphenols such as ginkgolic acids have been reported to induce contact dermatitis;8,9 cytotoxicity was also reported for these compounds.10

Extracts of G. biloba were reported to behave as an antiasthmatic,11 scavenge radicals,12 reduce cerebral insufficiency,13 alleviate the symptoms of mild to moderate Alzheimer-type dementia,13 and improve short-term memory in healthy subjects.14,15 While numerous investigations have attempted to determine the effects of the individual constituents of G. biloba extracts on the central nervous system,16 this aspect is still not well understood. Ginkgolide B (GB, BN 52021), a minor component of EGB 761, is a potent inhibitor of the platelet-activating factor (PAF) receptor.17,18 While PAF has been linked to long-term potentiation (LTP)19 and an increase of intracellular Ca2+ in the retina,16 this aspect is still not well understood. BB, the predominant terpene trilactone present in the leaves, has been reported to strongly inhibit phospholipase 2 (PLA2) activity in the brain and may contribute to the anti-ischaemic activity of EGB 761.21

An efficient and economical extraction of terpene trilactones is essential to secure the amounts of materials required for bioorganic and other studies. In contrast to conventional isolation procedures, which are tedious and lengthy,22 the method described below concentrates the terpene trilactone in the leaves (ca. 0.2%) to 70% in three rapid steps of oxidation, extraction, and washing. The clear advantage, at least at the laboratory scale, is that the extraction is shortened from several days to a single day because of minimal emulsion formation in the EtOAc extraction process and that only one organic solvent is used. The resulting extracts are ideal as starting material for pure terpene trilactone preparation from leaves, for enriching terpene trilactone content in nutraceuticals, and for providing low-flavonoid/high-terpene trilactone controls in medicinal studies. The four ginkgolides (ginkgolides A, B, C, J) and bilobalide isolated from the extract were identical in all respects with authentic specimens.
tion of BB (which is much less stable than the ginkgolides), but the amounts of ginkgolides also decreased, probably due to absorption onto solid MnO₂. Further investigations were focused on H₂O₂, as this was the only oxidizing agent that provided clear layer separation during extraction, while the other agents resulted in emulsions with lower recovery of terpene trilactones. Presumably the H₂O₂ treatment destroys the ingredient(s) that give rise to the tenacious emulsification in the EtOAc extraction step.

The concentration of H₂O₂ was varied to determine the optimal concentration. After washing, the total amount of extract decreased with higher percentages of H₂O₂, but without washings the amount of extract increased. Good separation of layers was found for 5% and 10% H₂O₂, whereas 1% H₂O₂ gave an emulsion and 3% H₂O₂ required more time for separation. The highest recoveries of terpene trilactones after washing were found with 3% and 5% H₂O₂. After extraction, H₂O₂ was detected in the organic layer (by the color change on filter paper impregnated with potassium iodide), while with 10% H₂O₂, the excess peroxide gave rise to an exothermic reaction upon workup. Therefore 5% H₂O₂ was used in all further investigations. With respect to the boiling time, 1 h was found to be optimal.

The Bioginkgo powder as well as leaves gave rise to acidic water (pH 3) after boiling. Since peroxide oxidation should work better under acidic conditions, oxidation with 1% of several acids and 5% H₂O₂ was tested. The results were either no change or decreased recovery of terpene trilactones. Thus the use of aqueous H₂O₂ without additional acid was found to be optimal.

After boiling with peroxide, all mixtures were extracted thrice with EtOAc. With respect to the number of extractions, three turned out to be sufficient. In the case of leaf extractions, a precipitate present in the organic layer had to be removed by filtration. Washing of the EtOAc layer with basic solutions was critical for high terpene trilactone content. Base was used to neutralize organic acids and to destroy peroxide excess; however, the lactone rings could open under such treatment, resulting in increased water solubility. For GB it was reported that lactones start opening at pH 6.5.24 As expected, aqueous Na₂CO₃ (pH 10.4) and NaSH (pH 10.8) resulted in lower recovery yields of terpene trilactones, with BB and ginkgolide C (GC) no longer being detected in the extract. However, washing with saturated solutions of NaHCO₃ and Na₂S₂O₃ (both pH 8.3) and Na₂SO₃ (pH 9.3) led to consistent extraction yields and high terpene trilactone content. Further workup yielded extracts containing 50–70% terpene trilactones. In contrast to basic solutions, use of aqueous NH₄Cl (pH 5) was not effective in removing impurities.

Since the lactones of terpene trilactones are closed at pH 3, the presence of any terpene trilactone in the discarded aqueous layer after washing with Na₂SO₃ (pH 9.3) was tested after acidification of the combined water layers with 1 N HCl; no terpene trilactones were detected by TLC. Using the same procedure for the water layers after washing with Na₂CO₃ (pH 10.4), GC and decomposition products of BB were found.

A typical extract prepared from Bioginkgo 27/7 is shown in Figure 3A. Small quantities of pure terpene trilactones were obtained from such extracts with semipreparative reversed-phase HPLC;25,26 larger quantities can be purified by a number of different procedures.25,26

After optimization, the protocol was applied to dried leaves. The total terpene trilactone contents were similar for the treated Bioginkgo 27/7 powder (65%), the yellow leaves (65%), and the green leaves (70%) (Figure 3).

The content of terpene trilactones in leaves depends on the season and other factors such as tree age.27 As described for leaves and for EGb761 (26% flavonoids, 6% terpene trilactone), BB was always the major component of the terpene trilactone content (ca. 50%).28,29 The procedures described above led to minimal BB destruction and high recovery yield and maintained a natural distribution pattern.

These leaf extracts possessed a significant amount of the undesired ginkgolic acids. The ginkgolic acids were removed by reversed-phase chromatography.30 The contents of the two predominant ginkgolic acids found in G. biloba leaves were measured by HPLC using PDA detection; in each case measured, the individual ginkgolic acids were present in amounts lower than the limit of quantitation (10 ppm).

**Experimental Section**

**General Experimental Procedures.** ¹H and ¹³C spectra were acquired on either a 300 or 400 MHz Bruker NMR using standard pulse sequences and parameters; further details are described below. Negative ion FABMS data were determined using a MS-HX110 tandem mass spectrometer (J EOL, Tokyo, Japan) instrument using a Xe beam (6 kV) with a 10 kV acceleration voltage and m-nitrobenzyl alcohol (NBA) matrix. Analytical HPLC measurements were performed on a Waters 996 PDA system with the Millenium Version 2.15.01 software package. Concentration graphs and linear regression were determined with Microsoft Excel 2000. Semipreparative HPLC isolations were achieved on a LC-908 (J apan Analytical Industry Co., Ltd.) instrument equipped with internal refractive index and ultraviolet detectors and a recorder.

**Extraction Material.** Bioginkgo 27/7 brand G. biloba extract was generously donated by Pharmanex (Provo, UT).

**Plant Material.** The leaves were collected from a female G. biloba tree in Morningside Park, New York, NY. The yellow fallen leaves were collected from the ground in November 2000.
while green leaves were collected from the tree in June 2001. They were frozen in liquid nitrogen, crushed, and lyophilized until constant weight. The moisture content was 59% in the green leaves and 73% in green leaves. Further samples of these leaves are stored in our laboratory.

**General Extraction of Terpene Trilactones.** Bioginkgo 27/7 extract (25 g) or lyophilized leaves (250 g) were boiled for 1 h in 0.5 L (extract) or 2 L (leaves) of 5% aqueous H2O2 (or other oxidation reagents in water). After passing (extract until constant weight. The moisture content was 59% in the green leaves.

Concentration gave an off-white amorphous powder with 60–70% content of terpene trilactones. A more rigorous procedure involved dissolving the extracts in MeOH, adding activated charcoal, and filtering over Celite 545.

**TLC Analysis of Terpene Trilactones.** Thin-layer chromatography of terpene trilactones was performed as previously reported.

**HPLC Separation of Terpene Trilactones.** A 1 × 25 cm 5 μm YMC ODS-AM column eluted with a 1 mL/min H2O/MeOH/TFA (7:2:1) solvent system was used to separate the terpene trilactones; monitoring was performed with a refractive index detector.

**Removal of Ginkgolic Acids.** Ginkgolic acids were removed from the extracts by reversed-phase chromatography using polymeric resins such as Diaion HP-20 or Amberlite XAD-16 with a step gradient of increasing methanold (30–90%) in water. Terpene trilactone-containing fractions (40–80%, as determined by NMR or TLC) were combined, and the solvent was removed to afford the final products. WP C18 silica was also employed (twice) with aqueous 60% methanol successfully to remove ginkgolic acids.

**Extraction of Ginkgolic Acids.** Standards were isolated from hexane-extracted G. biloba leaves that were subjected to chromatography with silica gel (1% MeOH in CHCl3), RP-18 silica gel (0–100% MeOH, retaining the 100% MeOH fraction), and semipreparative HPLC (1 × 25 cm 5 μm YMC ODS-AM column, H2O:MeOH:AcOEt (100:10:1) solvent system, monitored at 215 μm). Spectroscopic analyses of these compounds (H NMR, 13C NMR, and negative mode FABMS) provided results similar to those previously reported.

**Quantitation of Ginkgolic Acids.** Ginkgolic acid standards (3, 5, 25, 50, 250 μg) in MeOH (5 μL) were injected onto a 4.6 × 150 mm Phenomenex Luna 5 μm C18(2) column with 0.1% TFA in a MeCN solvent system (flow rate = 1 mL/min) and detection at 215 nm; concentration graphs showed good linearity ($R^2 ≥ 0.99$). Multiple injections determined the level of quantitation to be 10 ppm. Samples were prepared by dissolving 1–2 mg of extract in 100 μL of MeOH; 20 μL injections were then analyzed in a similar fashion.

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**References and Notes**

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