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Semisynthetic Preparation of Amentoflavone: A Negative Modulator at $GABA_A$ Receptors

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Abstract—Amentoflavone is found in a number of plants with medicinal properties, including *Ginkgo biloba* and *Hypericum perforatum* (St. John's Wort). We have developed a rapid and economic semi-synthetic preparation of amentoflavone from biflavones isolated from autumnal *Ginkgo biloba* leaves. Several studies have shown that amentoflavone binds to benzodiazepine receptors. Using two electrode voltage-clamp methodology, amentoflavone has been shown to be a negative modulator of GABA at $GABA_A$ $\alpha_1\beta_2\gamma_{2L}$ receptors expressed in *Xenopus laevis* oocytes This action appears to be independent of the flumazenil-sensitive benzodiazepine modulatory sites on the $GABA_A$ receptor.

Many recent studies indicate that flavones, a class of flavonoids, bind to the benzodiazepine site at $GABA_A$ receptors and exert anxiolytic activity in mice without many of the unwanted effects associated with the use of benzodiazepines.[1] Amentoflavone, a member of the biflavone class is a minor constituent of the herbal antidepressant St. John's Wort (0.01–0.05%)[2] and has been described as a high affinity inhibitor ($K_i = 6$ nM) of the benzodiazepine agonist ^{3}H-flunitrazepam, binding in a mixed type competitive and non-competitive manner to benzodiazepine receptors in synaptosomal membranes of the mammalian brain.[3] Additionally, amentoflavone has been reported to show a high affinity for human cloned benzodiazepine receptors ($K_i = 6$ nM)[4] and inhibit ^{3}H-flunitrazepam binding to rat brain ($IC_{50} = 14.9 \pm 1.9$ nM).[5] Based on the similar potency in displacing ^{3}H-diazepam binding in hippocampal and cerebellar membranes, it was concluded that amentoflavone does not discriminate between benzodiazepine receptor subtypes.[3]

Amentoflavone was the first high affinity ligand known for the benzodiazepine receptor devoid of nitrogen in its structure, and its biochemical characterisation led to the proposal that it behaves as a partial agonist. However, due to reported in vivo pharmacological efffects[6] and no change in the in vivo binding of ^{3}H-flunitrazepam being observed after intravenous administration of amentoflavone,[3] it has been suggested that it is either rapidly metabolised or cannot cross the blood–brain barrier.[3,6]

Recently amentoflavone has been found to be taken up into porcine brain endothelial cells predominantly by passive diffusion and is transported across porcine brain capillary endothelial cells (BCEC) monolayers, suggesting that amentoflavone would be able to penetrate the brain in vivo.[7] A comprehensive battery of in vitro radio-ligand binding assays has also shown that amentoflavone significantly inhibits binding at serotonin (5-$HT_{1D\alpha}$ $K_i = 4\,094$ nM, 5-HT_{2C} $K_i = 2\,555$ nM), D3-dopamine ($K_i = 1\,241$ nM), δ-opioid ($K_i = 36.5$ nM) while having no effect on GABA binding at the $GABA_A$ receptor site.[4] However, until now no studies have been carried out to determine the action of amentoflavone in functional GABA receptor assays.

The concentration of biflavones (Fig. 1) in *Ginkgo* leaves has been reported to vary from 0.047–1.68%, with amentoflavone being approximately 2% of the total biflavones.[8,9] The biflavones present in *Ginkgo* leaves, are all related compounds varying only in the

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presence of a methoxyl or hydroxyl group at positions 5, 3′ and 4‴, with the exception of 5″-methoxybilobetin, which has an additional methoxyl at the 5′ position. As a rapid and economic preparation of amentoflavone we have isolated the major biflavone component excluding 5′-methoxybilobetin of *Ginkgo biloba* leaves and subsequently carried out a demethylation to yield amentoflavone.

The biflavone content of *Ginkgo biloba* leaves increases from an undetectable quantity in the buds with low quantities appearing later, in the young leaves and the amount increasing slowly during the vegetative cycle, the highest amounts being found in fallen yellow autumnal leaves.[10] We used fallen autumnal leaves collected and air dried during April (southern hemisphere). Previously described methods for the isolation of amentoflavone[8] and 5′-methoxybilobetin[11] proved difficult and inefficient necessitating the development of improved methodology.

The dried autumnal yellow leaves were milled to a fine powder and first extracted twice with hexanes to remove non-polar components. ^{1}H NMR analysis of the components extracted by a number of solvents demonstrated that ethyl acetate was most effective at extracting the biflavones with the least amount of unwanted components. The crude biflavone extract was purified using silica gel and affinity chromatography to yield pure biflavones (1440 mg from 450 g of dried leaves) which was identified as approximately a 1:1 mixture of ginkgetin and isoginkgetin, with no sign of 5′-methoxybilobetin or any of the other biflavones known to be present in *Ginkgo biloba* leaves. This is consistent with a similar method of extraction that also reported isolating a mixture of ginkgetin and isoginkgetin.[12]

The mixture of ginkgetin and isoginkgetin was demethylated using hexadecyltributylphosphonium bromide in refluxing hydrogen bromide.[13] The reaction was monitored periodically by ^{1}H NMR which indicated rapid demethylation of the C-7 and C-3‴ methoxyls and much slower hydrolysis of the sterically shielded C-4′ methoxyl. Complete hydrolysis of all methoxyls occurred after 60–72 h. The crude product was purified using gel affinity chromatography and recrystallisation, to give amentoflavone[14] (87.5%) (>98% purity by ^{1}H NMR) with identical spectrometric data to that previously reported.[8]

The electrophysiological action of amentoflavone was investigated using two-electrode voltage clamp recording of human recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes[15] according to previously reported methods.[16] Amentoflavone alone produced no response, however when amentoflavone was tested in the presence of GABA (30 μM) it was found to be a negative modulator of the GABA response ($\alpha_1\beta_2\gamma_{2L}$) (Fig. 2). Amentoflavone was found to be a moderate negative modulator of GABA with an $EC_{50} = 3.6$ μM (95% C. I. $2.89 < m < 4.46$) ($\log IC_{50} = 0.55 \pm 0.05$) (Fig. 3).

This inhibitory action of amentoflavone on GABA responses was unaffected by increasing concentrations of flumazenil (0.1–100 μM) (results not shown), indicating

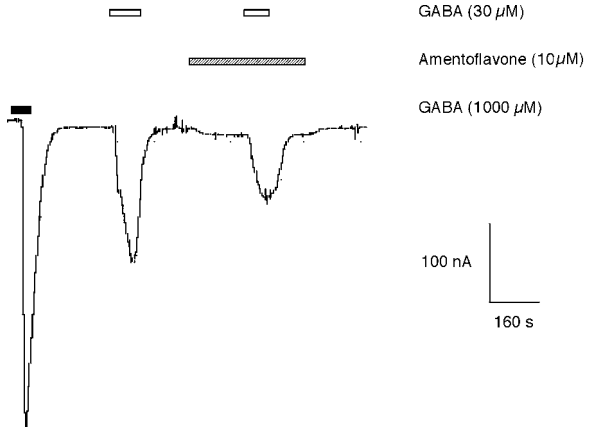
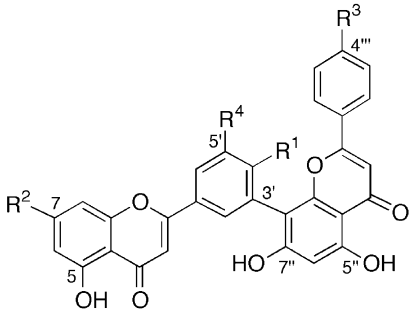


Figure 2. Current trace from an oocyte expressing $GABA_A$ $\alpha_1\beta_2\gamma_{2L}$ receptors in the presence of GABA (30 and 1000 μM) and amentoflavone (10 μM), showing inhibition of the GABA (30 μM) response by amentoflavone (10 μM).



Biflavone	R^1	R^2	R^3	R^4
Amentoflavone	OH	OH	OH	H
Bilobetin	OCH_3	OH	OH	H
Ginkgetin	OCH_3	OCH_3	OH	H
Isoginkgetin	OCH_3	OH	OCH_3	H
Sciadopitysin	OCH_3	OCH_3	OCH_3	H
5'-Methoxybilobetin	OCH_3	OH	OH	OCH_3

Figure 1. Structures of biflavones in *Ginkgo biloba*.

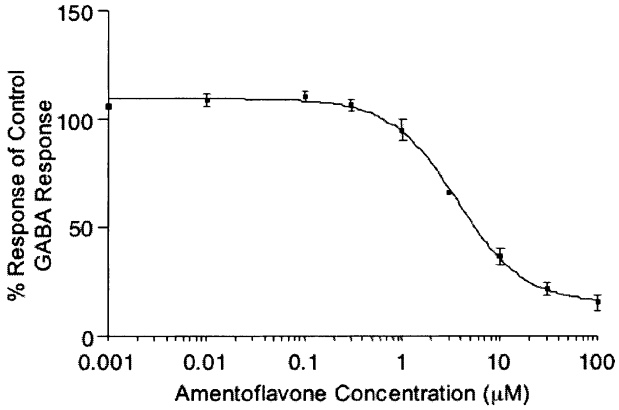


Figure 3. Dose–response curve for amentoflavone in the presence of 30 μM GABA at $GABA_A$ $\alpha_1\beta_2\gamma_{2L}$ receptors expressed in *Xenopus laevis* oocytes, $\log IC_{50} = 0.55 \pm 0.05$.

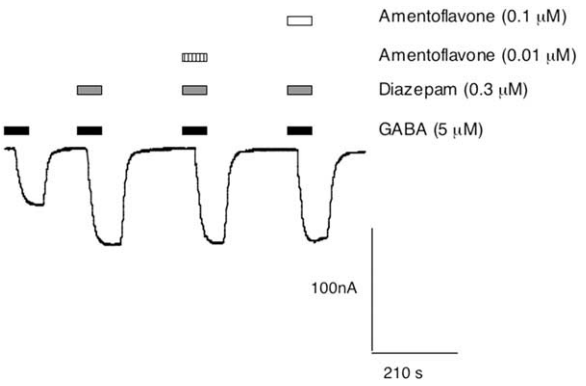


Figure 4. Current trace from an oocyte expressing $GABA_A$ $\alpha_1\beta_2\gamma_{2L}$ receptors in the presence of GABA (5 μM), Diazepam (0.3 μM) and Amentoflavone (0.01 and 0.1 μM). Diazepam (0.3 mM) enhances GABA (5 μM) response, amentoflavone (0.01 and 0.1 μM) has no effect on this enhancement.

that the negative modulation of $GABA_A$ ($\alpha_1\beta_2\gamma_{2L}$) receptors by amentoflavone is not mediated via high affinity (flumazenil sensitive) benzodiazepine sites. Additionally, at concentrations of amentoflavone below 0.1 μM in the presence of GABA (5μM) and diazepam (0.3 μM), amentoflavone has no effect on the enhancement of the GABA response by diazepam (Fig. 4) offering further evidence that the negative modulation of $GABA_A$ receptors by amentoflavone is independent of high affinity benzodiazepine binding sites.

We have developed a high yielding, rapid semisynthetic method for the preparation of amentoflavone and we have carried out the first electrophysiological investigation of amentoflavone at functional $GABA_A$ ($\alpha_1\beta_2\gamma_{2L}$) receptors. Amentoflavone acts as a negative modulator of the $GABA_A$ response. This action is unlikely to occur via a direct action at the GABA binding site since amentoflavone has been reported not to affect GABA binding.[4] In addition the action of amentoflavone at $GABA_A$ receptors does not appear to be mediated by high affinity (flumazenil sensitive) benzodiazepine sites, despite being shown to bind with high affinity at these sites.[4–6]

Acknowledgements

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14. **Experimental.** ^{1}H NMR spectra were recorded at 300 MHz using a Varian Gemini 300 instrument. Chemical shifts (δ_H) are quoted in parts per million (ppm), referenced internally to tetramethylsilane (TMS) at 0ppm. Coupling constants (J) are reported in Hertz. ^{13}C NMR spectra were recorded at 75.46 MHz on a Varian Gemini 300 instrument. Chemical shifts (δ_C) are quoted in ppm, referenced to acetone-d_6 at 29.92 ppm. Low resolution mass spectra were recorded on a Finnigan/MAT TSQ 7000 LCMS/MS spectrometer; only molecular ions (M^+) and major peaks are reported, with intensities quoted as percentages of the base peak. High resolution mass spectra were recorded using electrospray ionisation (ESI) on a Micromass QTof II at The Biomolecular Mass Spectrometry Laboratory, Department of Chemistry, University of Wollongong. Thin layer chromatography (TLC) was performed on aluminium backed plates pre-coated with silica (0.2 mm, $60F_{254}$) which were developed using UV fluorescence (254 nm) and alkaline $KMnO_4$ solution (0.5% w/v). Flash vacuum chromatography was performed on silica gel (Merck silica gel 60H particle size 5–40 μm). Chemicals were purchased from Sigma-Aldrich at the highest grade available.

Isolation of biflavones. Desiccated finely milled yellow gingko leaves (150 g) were extracted with hexane (1.8 L) for 48 h. The leaves were filtered and air dried for 1 h. The leaves were subsequently extracted with ethyl acetate for 24 h. The leaves were filtered and discarded. Removal of ethyl acetate under reduced pressure yielded a green precipitate (3.685–3.705 g). The precipitate was dissolved in chloroform (10–15 mL) and chromatographed over a silica gel column using chloroform-methanol mixtures (2–5% methanol in chloroform; 400 mL). Fractions containing biflavones were combined and evaporated to dryness (1.206–1.938 g). The precipitate was dissolved in acetone-methanol (1:1, 10–15 mL) and purified by chromatography on a Sephadex LH-20 gel column (2 cm internal diameter) using methanol as the eluting solvent. Fractions containing biflavones were combined and evaporated to dryness yielding a yellow precipitate (448–514 mg). ^{1}H NMR analysis showed a mixture of two biflavones, isoginkgetin and ginkgetin. Biflavonoid mixture ^{1}H NMR (acetone-d_6, δ_H, I and G denote isoginkgetin and ginkgetin, respectively) 3.81 (3H C4′-OCH_3), 3.87 (6.26 (3H C7′-OCH_3, I + G), 3.91 (3H C4‴-OCH_3, I), 6.23 (1H, d, J = 2.5 Hz, H-6 I), 6.31 (1H, d, J = 2.5 Hz, H-6 g), 6.46 (2H overlapping, d, J = 2.4 Hz, H-6″), 6.52 (1H, d, J = 2.4 Hz, H-8, I), 6.69 (1H, s,

H-3″ G), 6.76 (1H, d, $J=2.4$ Hz, H-3, G), 6.81 (2H, d, $J=8.6$ Hz, H-3′ and H-5′, G), 6.83 (1H, s, H-3, I), 6.92 (2H, d, $J=8.8$ Hz, H-3‴ and H-5‴, I), 7.35 (2H, overlapping d, $J=8.6$ Hz, H-5′ I + G), 7.55 (2H, d, $J=8.6$ Hz, H-2‴ and H6‴, G), 7.66 (2H, d, $J=8.6$ Hz, H-2‴ and H6‴, I), 8.14 (2H, overlapping d, $J=2.4$ Hz, H-2′, I + G) and 8.17 (2H, overlapping dd, $J=2.4$, 8.8 Hz, H-6′ I + G).

Amentoflavone Biflavones (448 mg) were dissolved in 48% hydrogen bromide (200 mL), hexadecyl-tributylphosphonium bromide (50 mg) was added and the reaction mixture refluxed for 72 h, with samples taken periodically for monitoring by ^{1}H NMR spectroscopy. Once demethylation was complete, the solvent was removed under reduced pressure. The precipitate was dissolved in water (200 mL), and was extracted with ethyl acetate (5×200 mL) the combined organic extracts were dried over anhydrous sodium sulfate and evaporated to dryness. The precipitate was dissolved in acetone-methanol (1:1, 15 mL), applied to a Sephadex LH-20 gel column (2 cm internal diameter) and eluted with methanol. Fractions containing biflavones were combined, evaporated under reduced pressure and recrystallisation from ethyl acetate yielding amentoflavone as a yellow amorphous powder (373 mg, 87.5%). Melting point: 254–256 °C. lit. (255–256 °C)[8] ^{1}H NMR (acetone-d_6, δ_H): 6.26 (1H, d, $J=2.5$ Hz, H-6), 6.46 (1H, d, $J=2.4$ Hz, H-6′), 6.54 (1H, d, $J=2.4$ Hz, H-8), 6.69 (1H, s, H-3′), 6.76 (1H, s, H-3), 6.85 (2H, d, $J=8.8$ Hz, H-3′' and H-5′'), 7.27 (1H, d, $J=8.6$ Hz, H-5′), 7.68 (2H, d, $J=8.8$ Hz, H-2″ and H6″), 8.06 (1H, q, $J=2.4$ Hz and 8.6 Hz, H-6′) and 8.16 (1H, d, $J=2.4$ Hz, H-2′). ^{13}C NMR (d_6-acetone, δ_C): 94.93 (C-6″), 99.67 (C-8), 99.92 (C-6), 101.92 (C-3″), 104.47 (C-3 + 5a), 105.51 (C-8″), 105.71 (C-5″a), 116.85 (C-3‴ + 5‴), 117.66 (C-5′), 120.94 (C-1‴), 123.44 (C-1′), 123.58 (C-3′), 128.97 (C-6′), 129.26 (C-2‴ + 6‴), 132.74 (C-2′), 156.26 (C-8″a), 158.97 (C-5″ + 8a), 160.36 (C-5), 161.93 (C-4′), 162.67 (C-7″), 162.93 (C-4‴), 163.49 (C-2), 164.99 (C-2″), 165.23 (C-7), 183.19 (C-4″) and 183.58 (C-4). MS (APCI) m/z 538 (100%) (M^+), ESI 539.0990 (MH^+ $C_{30}H_{19}O_{10}$ requires 539.0978).

15. Human α_1, β_2 and γ_{2L} cDNAs subcloned in pcDM8 (Stratogene, La Jolla, CA, USA) were kindly provided by Dr. Paul Whiting (Department of Biochemistry and Molecular Biology, Neuroscience Research Centre, Merck Sharp and Dohme Research Laboratories, Harlow, Essex, UK). Drug solutions were prepared by diluting 100 mM aqueous stock solutions of GABA and 100 mM DMSO stock solutions of amentoflavone and diazepam in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$–$6H_2O$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5). The highest concentration of DMSO superfusing the oocytes was 0.8%, at which concentration DMSO had no effects. Expression of $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors in *Xenopus laevis* oocytes and two-electrode voltage-clamped electrophysiological recording was carried out as previously described.[14] The procedures involved in the use of *X. laevis* were approved by the Animal Ethics Committee of the University of Sydney.

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