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Xanthones from *Gentiana campestris* as New Acetylcholinesterase Inhibitors

Aurélie Urbain, Andrew Marston, Emerson Ferreira Queiroz, Karine Ndjoko, Kurt Hostettmann

Abstract

In order to discover new acetylcholinesterase (AChE) inhibitors, different plant extracts were screened by a previously established TLC bioautographic method. The methanol extract of *Gentiana campestris* leaves exhibited significant inhibition of AChE activity. A bioactivity-guided fractionation approach was undertaken to isolate the active components. Four xanthones, bellidin, bellidifolin, bellidin 8-*O*- β -glucopyranoside (norswertianolin), and bellidifolin 8-*O*- β -glucopyranoside (swertianolin), were found to be responsible for the anti-AChE activity effects. Bellidifolin showed similar activity to galanthamine in this enzyme assay.

Alzheimer's disease is the most common age-related degenerative brain disorder, gradually and irreversibly evolving in a loss of memory and other mental abilities [1]. A common approach for treating this disease is to enhance the acetylcholine level in the brain using acetylcholinesterase inhibitors [2]. In addition to the synthetic inhibitors tacrine, donepezil and rivastigmine, plant-derived inhibitors, such as galanthamine and huperzine A, are also effective [3]. Galanthamine hydrobromide is now approved for the treatment of Alzheimer's disease in several countries. Until now, natural inhibitors of AChE have mainly been alkaloids [3], [4]. Certain other compounds have been shown to exhibit a weaker anti-AChE activity, e.g., monoterpenoids [5], [6] and phenolic compounds [7].

In an effort to discover new, non-alkaloid inhibitors of AChE, a screening program was undertaken, using a TLC bioautographic assay [8]. In this method, the plant constituents are first separated by TLC. Then AChE is sprayed on the plate. After incubation at 37 °C in order to reactivate the enzyme, a mixture of 1-naphthyl acetate and Fast Blue B salt is sprayed on the TLC plate. Active enzyme cleaves 1-naphthyl acetate to yield 1-naphthol, which in turn reacts with Fast Blue B salt to give a purple-coloured diazonium dye. AChE inhibitors on the plate prevent the enzyme from

Dedicated to Professor Adolf Nahrstedt on the occasion of his retirement as Editor-in-Chief of *Planta Medica*

Affiliation: Laboratory of Pharmacognosy and Phytochemistry, Geneva-Lausanne School of Pharmacy, University of Lausanne, Lausanne, Switzerland

Correspondence: Prof. Kurt Hostettmann · Laboratory of Pharmacognosy and Phytochemistry · Geneva-Lausanne School of Pharmacy · University of Lausanne · BEP · 1015 Lausanne · Switzerland · Phone: +41-21-692-4561 · Fax: +41-21-692-4565 · E-mail: Kurt.Hostettmann@pharm.unige.ch

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reacting with 1-naphthyl acetate, and from producing the purple colouration: so the areas corresponding to inhibitory compounds show up as white spots against a purple background.

Out of the 50 plant extracts investigated, a methanol extract of *Gentiana campestris* L. (Gentianaceae) leaves was shown to contain several compounds inhibiting the AChE activity.

The crude extract was first analysed by LC/UV. On finding suitable separation conditions, LC/microfractionation was performed on the methanol extract. A part of each fraction was then submitted to the bioautographic assay. Three compounds (**1–3**) were found to inhibit the enzyme. This allowed attribution of activity to peaks in the chromatogram and showed the three active compounds to be xanthenes on the basis of their typical UV spectra. A fourth compound (**4**), the major product in the extract, had a UV spectrum very similar to those of the active xanthenes, but did not inhibit AChE in initial testing. The identification of this constituent and the three anti-AChE xanthenes was undertaken, in order to study the relation between structure and activity. Previous phytochemical investigations of *G. campestris* have shown the presence of tetraoxygenated xanthenes with 1,3,5,8- and 1,3,7,8-oxidation patterns, and penta-oxygenated xanthenes with a 1,3,4,5,8-oxidation pattern [9].

LC/MS of the crude extract provided useful on-line complementary structural information for all peaks of interest (Fig. 1). Compound **2** exhibited an $[M + H]^+$ ion at $m/z = 261$ and was then identified as a tetrahydroxyxanthone; the $[M + H]^+$ ion at $m/z = 275$ suggested that compound **3** was a trihydroxymethoxyxanthone; the mass spectrum of **1** gave ions at $m/z = 437$ $[M + H]^+$ and 275 $[M + H - 162]^+$, and thus the structure was postulated to be an *O*-glycosyldihydroxymethoxyxanthone. The main xanthone **4** was identified as an *O*-glycosyltrihydroxyxanthone, on the basis of two ions at $m/z = 261$ $[M + H - 162]^+$ and 423 $[M + H]^+$.

In order to study the activity of each xanthone, they were isolated by semi-preparative HPLC. Four fractions corresponding to the peaks of interest were collected. LC/UV analyses showed that each fraction contained a pure compound. Xanthone **4** (10.0 mg) was obtained from fraction 1, xanthone **1** (6.0 mg) from fraction 2, fraction 3 afforded xanthone **2** (4.8 mg), and xan-

thone **3** (8.1 mg) was obtained from fraction 4. Their structures were confirmed by 1D and 2D NMR analyses and by comparison of the data with those of the literature [10], [11], and with previously isolated reference samples. Four xanthenes were isolated from *G. campestris* (Fig. 2): bellidifolin 8-*O*- β -glucopyranoside (**1**), bellidin (**2**), bellidifolin (**3**), and bellidin 8-*O*- β -glucopyranoside (**4**). These xanthenes were first identified in the species by Kaldas et al. [12].

The pure compounds were then tested against AChE using the bioautographic assay. A quantity of 1 μ g of each xanthone deposited on the TLC plate gave an inhibition spot after spraying with enzyme and substrate. All the xanthenes were thus active, even xanthone **4**, which was not inhibitory in the crude extract. Dilutions were then made in order to find the minimum quantity required to produce white inhibition spots (Table 1). It was found that all xanthenes inhibited the enzyme at less than 0.5 μ g. The two aglycones were more active than their glycosides. This could be due to hydrophobicity or steric factors that favour the inhibition mechanism. Furthermore, **3** was more active than **2**, just as **1** was more active than **4**, suggesting that the methoxy group in position C-3 enhances the activity.

Contrary to the prediction of Kang and Fang [13] that oxygenated polycyclic aromatic hydrocarbons, such as xanthenes, should have little effect on AChE activity, it is demonstrated here that this class of compound is, in fact, active and provides very good inhibitors. Indeed, bellidifolin, the most active of the four tested xanthenes, inhibited the enzyme in the bioassay as strongly as the commercial AChE inhibitor galanthamine.

For the moment, the use of inhibitors of acetylcholinesterase still seems to be the most efficient approach to treat the symptoms of Alzheimer's disease. However, current regimens present some disadvantages, such as a short half-life, or side effects [14]. For this reason, it is important to continue the search for new inhibitors. Tetraoxygenated xanthenes are very widespread in certain plant families, such as the Gentianaceae, and appear to be efficient inhibitors of acetylcholinesterase. A systematic study has now been undertaken with various *Gentiana* species; preliminary results show that xanthenes with other substitution patterns, such as 1,3,7,8-tetraoxygenated xanthenes from *G.*

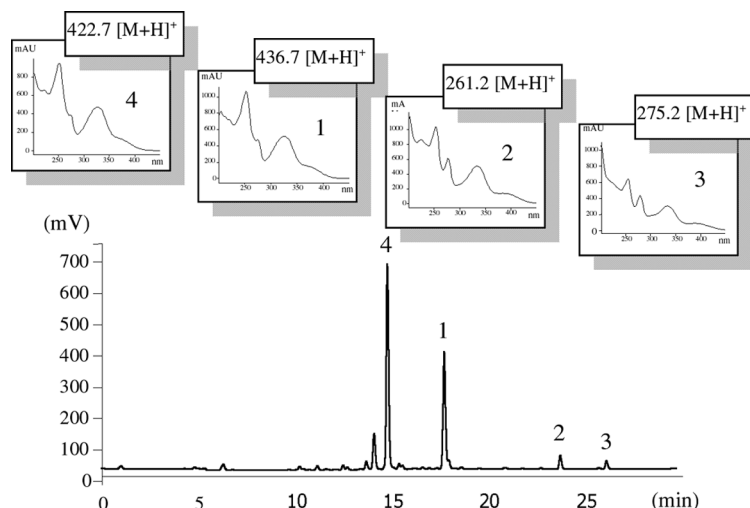
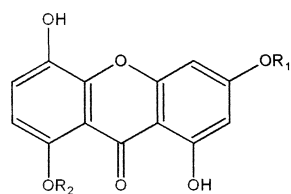


Fig. 1 HPLC-UV-MS analysis of a methanol extract of *G. campestris* leaves. Column Nova-Pak RP-18 (150 \times 3.9 mm, 5 μ m). Gradient: MeOH-H₂O + 0.05% TFA (5:95 \rightarrow 100:0, 30 min). Flow-rate: 1.0 mL.min⁻¹. Detection: UV 254 nm.



- 1:** R₁ = CH₃, R₂ = β-glucopyranosyl
2: R₁ = R₂ = H
3: R₁ = CH₃, R₂ = H
4: R₁ = H, R₂ = β-glucopyranosyl

Fig. 2 Tetraoxygenated xanthenes from *Gentiana campestris* L.

bavarica or 1,3,7,-trioxygenated xanthenes from *G. punctata*, also exhibit AChE inhibition. Isolation of these xanthenes is presently underway in order to investigate structure-activity relationships.

Before this class of non-alkaloidal metabolites can be considered as a new potential treatment for Alzheimer's disease, further *in vitro*, *in vivo*, and toxicological testing is necessary.

Materials and Methods

Gentiana campestris whole plants were collected at Col de Molendruz, VD, Switzerland, in September 1997. A voucher specimen (97067) is deposited at the Laboratory of Pharmacognosy and Phytochemistry, University of Lausanne. Powdered leaves (100 g) of *G. campestris* were extracted successively with 60 – 80°C petroleum ether (3×500 mL, 24 h), chloroform (3×500 mL, 24 h) and methanol (3×500 mL, 24 h) at room temperature, to give 1.8 g, 2.2 g, and 12 g of extract, respectively.

HPLC-UV was performed on a Hewlett-Packard 1100 instrument equipped with a photodiode array detector and a Nova-Pak RP-18 column (4 μm, 150×3.9 mm i.d., Waters). An MeOH-H₂O gradient (5 : 95 → 100 : 0) containing 0.05% TFA was applied at a flow rate of 1.0 mLmin⁻¹ during 30 min.

LC/microfractionation was performed on 3 mg of methanol extract, using an RCM 8×10 radial compression module (Waters) equipped with a Radial-Pak column filled with μBondapak C₁₈ (10 μm, 8×100 mm i.d., Waters), and a Shimadzu LC-10AD pump. The elution conditions were the same as for analytical

HPLC, except that the run time was extended to 60 min. The extract was monitored at 254 nm. Fractions were collected every minute (1 mL/fraction) with a Gilson FC 204 fraction collector in Eppendorf tubes. All the fractions were evaporated to dryness with a Speedvac system (Jouan) and then dissolved in 100 μL of MeOH. A part (2 μL) of each fraction was then submitted to the bioautographic assay. Three compounds were found to inhibit AChE activity, distributed over 7 fractions: fractions 36 to 39 (Rt: 36–40 min) contained compound **1**, fractions 48 and 49 (Rt: 48–50 min) contained compound **2**, and the third compound (**3**) was present only in the fraction 54 (Rt: 54–55 min). The purity of each compound was checked by HPLC/UV.

Fractionation of the main peaks was accomplished with Shimadzu LC-8A preparative pumps using a Waters PrepLC 25 mm radial compression module equipped with a PrepLC 25 mm column filled with μBondapak C₁₈ (10 μm, 25×100 mm i.d., Waters). The applied gradient was the same as for analytical HPLC, but with a flow rate of 10.0 mLmin⁻¹. Chromatograms were recorded using the same system as for LC/microfractionation. Four fractions corresponding to the peaks of interest were collected. Xanthone **4** (10.0 mg) was obtained from fraction 1 (Rt: 15.0 min), xanthone **1** (6.0 mg) from fraction 2 (Rt: 18.0 min), fraction 3 (Rt: 24.0 min) afforded xanthone **2** (4.8 mg), and xanthone **3** (8.1 mg) was obtained from fraction 4 (Rt: 26.3 min).

¹H- and ¹³C-NMR spectra were run on a Varian Unity Inova 500 MHz NMR instrument; compounds were dissolved in DMSO-*d*₆; TMS was used as internal standard.

The extract and the pure compounds were analysed under the same HPLC conditions as for analytical LC/UV. For LC/MS, a Finnigan LCQ detector was used, equipped with an atmospheric pressure chemical ionisation (APCI) interface as the ionisation source, which was operated under the following conditions: sheath gas: N₂ with flow of 77 L/min; capillary temperature: 150°C; APCI vapouriser temperature: 450°C; source voltage: 6 kV; source current: 5 μA; capillary voltage: 16 V; tube lens offset: 10 V; multipole 1 offset: -3.75 V; multipole 2 offset: -7.00 V; inter-multipole lens voltage: -10 V; trap DC offset voltage: -18 V. Full-scan mass spectra (150–500 amu) were recorded every 2 seconds in positive ion mode. The protonated molecule ions [M + H]⁺ of xanthenes were chosen as the parent ions for isolation and fragmentation. MSⁿ experimental conditions were as follows: He CID pressure: 0.5 mTorr; relative collision energy: 40%; fragmentation time: 200 ms. The spectra were acquired every second and the window was reduced to 50–500 amu.

The TLC bioautographic method in this study was the same as described previously [8]. Bellidin and bellidifolin were dissolved in EtOAc, whereas the respective glucosides were dissolved in MeOH. The solutions were applied to TLC in varying dilutions, and plates were developed with a mixture of CHCl₃-MeOH-H₂O (25 : 5:0.5). Three known inhibitors of acetylcholinesterase were used as reference compounds: huperzine A (product no. H 5777) and galanthamine HBr (product no. G 1660) were purchased from Sigma (St. Louis, MO, USA), physostigmine (eserine) was purchased from Fluka (Buchs, Switzerland; product no. 45710).

Table 1 Anti-acetylcholinesterase activity of tetraoxygenated xanthenes from *G. campestris* and reference inhibitors

Compound	Minimum inhibitory quantity [μg] ^a	[nmole] ^a
Bellidifolin (3)	0.01	0.03
Bellidin (2)	0.04	0.15
Bellidifolin 8-O-β-Glc (1)	0.08	0.18
Bellidin 8-O-β-Glc (4)	0.50	1.20
Galanthamine	0.01	0.03
Physostigmine	0.005	0.002
Huperzine A	0.002	0.0008

^a Minimum quantity required for observing a white inhibition spot on TLC.

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