Isolation of the Acetylcholinesterase Inhibitor Ungeremine from *Nerine bowdenii* by Preparative HPLC Coupled On-Line to a Flow Assay System

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In an attempt to isolate the active compound while detecting acetylcholinesterase inhibitory activity, we applied a fluorometric flow assay system to an on-line coupled preparative HPLC. The MeOH extract of *Nerine bowdenii* showed a strong inhibitory peak in the on-line assay, and the active compound was isolated by CPC and HPLC. It was identified as ungeremine by analysis of its 1H-NMR, 2D-NMR, and NOESY spectra. The assignment of the active *N. bowdenii* constituent was also confirmed by co-TLC, co-HPLC, and co-1H-NMR experiments using an authentic sample of synthetic ungeremine. The IC$_{50}$ value of ungeremine was 0.35 μM, showing stronger activity than galanthamine (2.2 μM).

**Key words** fluorometric; on-line HPLC; preparative HPLC; acetylcholinesterase inhibitor; ungeremine; *Nerine bowdenii*

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Inhibition of acetylcholinesterase (AChE) is a major treatment for Alzheimer’s disease. At the moment galanthamine is being used as an AChE inhibitor for the treatment of Alzheimer's disease, and is claimed to have less side effects than other known inhibitors like physostigmine and tacrine. Considerable efforts are being made to find good inhibitors from natural sources. To search for inhibitors from natural products, a selective and sensitive as well as simple method for screening is necessary. For the isolation of active compounds, a bioassay has to be carried out in each step of the isolation, which is a laborious step in most cases. If we can isolate the active compound while the activity is detected it would be very effective.

AChE activity has been detected using microplates or TLC colorimetrically and fluorometrically. Chemiluminescence imaging, which is even more sensitive, has also been used. AChE inhibitors has also been detected in a flow system photometrically, electrochemically, and fluorometrically, or by measuring pH changes. Flow injection analysis by fluorescence detection after electrophoretic separation of AChE inhibitors was reported. Recently, a colorimetric flow assay coupled on-line to HPLC for post column AChE activity detection has been reported.

Since HPLC is effective and widely used for the separation of various compounds from plant extracts, we combined a fluorometric flow assay with an HPLC on-line assay system to improve the sensitivity. The system could be set up using HPLC equipment without any other special devices. A preparative HPLC column was connected to the detection system, and this made isolation of the active compound simultaneously with activity detection possible.

Among the bulbs of amaryllidaceae plants screened previously for AChE inhibitory activity, *Nerine bowdenii* showed strong inhibition. In this experiment, the MeOH extract of *Nerine bowdenii* was analysed by an HPLC on-line activity detection system and the active AChE inhibitor was isolated using preparative scale centrifugal partition chromatography (CPC) and HPLC.

**MATERIALS AND METHODS**

**General Experimental Procedures** NMR spectra were recorded on a Bruker DMX-600 spectrometer, operating at 600 MHz. Electrospray ionization mass spectrum (ESI-MS) for on-line detection were measured on a Finnigan TSQ 700 and Finnigan MAT 900. UV spectra were recorded on a Varian Cary 1 Bio UV-visible spectrophotometer. A Bio-Rad Model 3550-UV microplate reader equipped with microplate manager software (version 4.0) was used for the microplate assay. A CPC (model LLB-M, Sanki Engineering, Tokyo, Japan) equipped with an LKB 2150 HPLC pump and an LKB Bromma 2211 Supercarr fraction collector was used for the large scale purification. For the HPLC on-line flow assay, a Lichrosopher 60 RP Select B (5 μm, 125×4 mm I.D.) HPLC column from Merck (Darmstadt, Germany) and a μ-Bondapack C18 preparative HPLC column (10 μm, 300×7.8 mm I.D.) from Waters (Milford, MA, U.S.A.) were used. An LKB type 2150 HPLC pump (Bromma, Sweden) was used for the delivery of eluent, and Minipuls 3 (Gilon, Villiers-le-Bel, France) low pressure pumps were used for the enzyme and substrate. A Waters 990 photodiode array (PDA) detector (Millipore (Milford, MA, U.S.A.) was connected to a Waters 5200 printer plotter for UV chromatogram. A Shimadzu RF-535 fluorescence HPLC monitor (Kyoto, Japan) connected to a pen recorder (Kipp & Zonen BD 41, The Netherlands) was used for fluorescence detection. PFA tubing (0.5 mm I.D., Upchurch Scientific, Oak Harbor, WA, U.S.A.) was used for reaction coil. For TLC, DC-Alufolien silicagel 60 F254, 0.2 mm thickness plates (Merck) were used. Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was used for purification of the isolated compound. Acetylcholinesterase from electric eel (type VI-s, lyophilized...
powder, 292 U/mg solid, 394 U/mg protein, Sigma Chemical Co.) was used. Acetylthiocholine iodide (ATCI) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and the standard compound galanthamine hydrobromide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 7-Acetoxy-1-methylquinolinium iodide (AMQI) from Fluka (Buchs and Switzerland) was used as fluorogenic substrate.

**Plant Material** The bulbs of *Nerine bowdenii* W.L. Watson were obtained from the bulb collection of J. Leenen b.v., Sassenheim, the Netherlands. The bulbs were stored at 4 °C until used.

**Extraction** *N. bowdenii* bulbs were chopped into small pieces using an Ultra Turrax grinder, freeze dried, and then extracted with 10 ml of methanol per gram of dry weight for 24 h at room temperature. The extract was decanted and the residue was extracted again with the same amount of fresh methanol for four more days. The two methanol extracts were combined and evaporated with a rotary evaporator. The MeOH extract was fractionated into hexane and a 50% MeOH phase. The 50% MeOH phase was evaporated in a rotary evaporator, and injected into the HPLC after redissolving it in 30% MeOH.

**Microplate Assay** The AChE inhibitory activity of ungeremine and galanthamine was measured quantitatively, using a 96-well microplate reader, based on Ellman's method. Both of the samples were dissolved in methanol. The absorbance was measured 10 times at 405 nm every 15 s before and after adding the enzyme. The reaction rate was calculated by Microplate Manager software (version 4.0), (Bio-Rad Laboratories). The percentage inhibition was calculated by comparing the rates for the sample to the blank (MeOH). To calculate the IC50 values using EXCEL®, each sample was assayed at three different concentrations in triplicate for each concentration, and the assay was repeated three times. The percent inhibition for galanthamine was found to be 33.8, 52.7, and 96.3% at 1.0, 2.5, and 10 μM, respectively. For ungeremine, it was 11.2, 23.8, and 62.2% at 0.05, 0.1, and 0.5 μM, respectively.

**TLC Assay** Enzyme inhibitory activities of the HPLC fractions were confirmed on silica gel TLC plates by spraying the substrate, dye and enzyme. Samples were spotted on the two TLC plates identically and developed in CHCl3-MeOH-H2O (6:4:1) at the same time. One plate was sprayed for TLC assay and the other was sprayed for false-positive detection. Five units/ml of enzyme solution was used for spraying. A yellow background appeared and white spots due to inhibitory compounds were visible after about 5 min.

**Detection of False-Positive Inhibition** False-positive inhibition could be detected as reported previously. Five units/ml of AChE was used to incubate the enzyme–substrate mixture for thiocholine spray. White spots on the yellow background were recorded and the results were compared with the results of TLC assay. If white inhibitory spots observed in the TLC assay were also seen as white spot in the false-positive activity assay, it was considered as false-positive activity. If white spots were seen only in the TLC assay, and not in the false-positive activity assay, it was considered to be true activity.

**Flow Assay System Coupled On-Line to HPLC and MS** The fluorescent flow assay system was coupled to HPLC and MS to isolate the active compound at the time of activity detection. The layout of the on-line system is shown in Fig. 1. The eluent of the mixture of MeOH–H2O–THF (30:69:1) with 0.01 M ammonium acetate was pumped in at a rate of 1 ml/min for the analytical HPLC column (Lichrospher 60 RP Select B, 5 μm, 125×4 mm I.D.), or 2 ml/min for the preparative HPLC column (μ-Bondapack C18 preparative HPLC column, 10 μm, 300×7.8 mm I.D.) using an HPLC pump. A manual injector with a 30 μl injection loop for the analytical column and a 100 μl injection loop for the preparative column were used. After the HPLC column, the flow was divided into three parts using a splitter; 40 μl/min into the assay system, 40 μl/min into the MS, and 920 μl/min to the photodiode array (PDA) detector to get the UV chromatogram. The PDA detector was connected to a printer plotter. When the preparative column was used, 80 μl/min went into the assay system and the rest was sent via the PDA detector to the fraction collector. A PEEK restriction capillary (64 μm I.D.) was used to regulate the flow rate into three parts by adjusting its length. A flow of 40 μl/min (or 80 μl/min) which goes to the assay system was mixed with a flow of 40 μl/min (or 80 μl/min) of enzyme (0.1 unit/ml in 50 mM sodium phosphate pH 7 with 0.1% BSA) and that of sub-

![Fig. 1. Layout of Flow Assay System Coupled On-Line with HPLC and MS](image-url)
strate (1.5 μM AMQI in 50 mM sodium phosphate–citrate buffer, pH 5) through 3-way joint pieces leading to the reaction coil (hand-knitted PFA tubing, inner volume 300 μl), and detected by a fluorescence monitor.

**Isolation of Active Compound** Initially the activity was detected in the on-line system with an analytical HPLC column by injecting 30 μl of *N. bowdenii* extract (10 mg/ml) using the eluent MeOH–H2O–THF (30 : 69 : 1) containing 0.01 M ammonium acetate. Then 100 μl of the extract (200 mg/ml) was injected into a preparative HPLC connected on-line to the assay system. This was repeated fifteen times. The active fraction was collected using the same eluent as in the analytical column. The active fraction was further purified with an analytical HPLC column eluted with MeOH–H2O–THF (30 : 68 : 2), containing 0.01 M ammonium acetate. The collected active compound was loaded on the Sephadex LH-20 column (60/1100 mm I.D.) and eluted with MeOH to remove the salts and impurities. The isolated compound was identified by the UV, MS, 1H-NMR, HMBC, and NOESY spectra by comparison of its spectral data to those of the reference compound (synthetic ungeremine TFA salt).

To obtain the active compound in a larger amount, 1 g of the extract was loaded on the CPC and separated using ethylacetate–MeOH–H2O (45 : 20 : 35), with the lower phase as the stationary phase and upper phase as the mobile phase. The active fraction identified by TLC was further separated by a preparative HPLC column at a flow rate of 2.5 ml/min, an analytical HPLC column at a flow rate of 1.2 ml/min repeatedly using MeOH–H2O–THF (30 : 68 : 2), and purified with a Sephadex LH-20 column (60×5 mm I.D.).

**Isolated Ungeremine** UV (MeOH) λ_{max} 260, 280, 365, and 416 nm; 1H-NMR (600 MHz, D2O with 0.01% TFA-d1) δ 9.19 (1H, s, H-7), 7.88 (1H, s, H-11), 7.59 (1H, s, H-8), 7.54 (1H, s, H-1), 7.34 (1H, s, H-3), 6.32 (2H, s, OCH2O), 5.15 (2H, t, J=7.0 Hz, H-5), 3.70 (2H, t, J=7.1 Hz, H-4); 1H–1H-COSY (proton correlated) H-7 (H-5, H-11, H-8), H-11 (H-7, H-8), H-8 (H-7, H-11), H-1 (H-3, H-4), H-3 (H-4, H-1), H-5 (H-4, H-7), H-4 (H-5, H-3, H-1); NOESY H-7 (H-8), H-8 (H-7, H-4), H-11 (H-1).

**RESULTS AND DISCUSSION**

**HPLC On-Line Flow Assay** Because of the solvent influence on the enzyme activity in the flow assay system(1,2) when the assay system was connected to an HPLC using 30% MeOH, 1% tetrahydrofuran (THF), and 0.01 M ammonium acetate as eluent, the detection limit for galanthamine was 0.15 nmol, still about 6 times more sensitive than the HPLC on-line colorimetric assay (0.9 nmol),(3) although this was not as sensitive as we expected when we tried to develop the fluorescent method. It seems that the low pH and low temperature necessary for the stability of the substrate result in lower enzyme activity and thus lower sensitivity.

**Active Compound from Nerine bowdenii** When the extract of *N. bowdenii* was injected into an analytical HPLC column (Lichrospher 60 RP Select B, 5 μm, 125×4 mm I.D.) connected to the flow assay system, a broad inhibitory peak was observed at about 17.5 min (Fig. 3). By using galanthamine as the internal standard, the active peak in the biological activity detection could be recognized in the UV chromatogram (B) and in the MS spectrum (C). Thirty microliters of a 1 : 1 mixture of 1 mM galanthamine and a 50 mg/ml sample in 30% MeOH was injected. The galanthamine peak could be recognized by comparing the chromatograms of extract alone (Fig. 3) with galanthamine added (Fig. 4). Galanthamine showed activity at 9.33 min in the biological activity detection (Fig. 3A) at 5.33 min in the UV chromatogram (B), and at 6.6 min in MS (C). A 4 min difference between the activity peak and the UV chromatogram peak of galanthamine suggests that the active peak of the extract correlates with the one around 13.5 min in the UV chromatogram. The 2.7 min difference between the activity peak and
MS, and the 1.3 min difference between the UV chromatogram and MS of galanthamine tells us that the active peak of the extract seems to be around 14.80 min in the MS. Actually we could recognize a peak at 13.70 min in the UV chromatogram, and at 14.85 min (m/z=266, unknown compound) and 14.92 min (m/z=266 [M+H]^+ of ungeremine) in the MS spectrum of the extract.

When a preparative column (μ-Bondapack C18, 10 μm, 300×7.8 mm I.D.) was used with the flow assay system, a broad activity peak was observed at about 65—69 min. It seems to correspond to the one at 63—66 min in the UV chromatogram. Since the peaks were broader than in the analytical column, the nearby fractions were also collected and tested for activity by TLC assay and it was confirmed that the peak at 65 min was the active one. The active fraction was collected repeatedly using a total of 300 mg of extract. The active fractions were evaporated in a rotary evaporator and re-injected into the analytical HPLC column for purification. This showed four peaks in the UV chromatogram, and each was analyzed for activity and false-positive activity by the TLC assay (Fig. 5). Peak number 2 showed a white inhibitory spot on the TLC assay (B) but no false positive spot (C), and was thus proven to be the real active compound, and further collected. Its UV spectrum showed maximum absorbance at 260 nm (A). This active compound was loaded in a small Sephadex LH-20 (60×5 mm I.D.) column to remove the salts and impurities, and analyzed by NMR and MS. MS analysis of this purified compound showed a [M+H]^+ of 266. The NMR spectrum was in agreement with that reported for ungeremine (Fig. 2), except for the signals at δ 7.64 and δ 7.68. These signals appeared to be switched with each other when compared to the NMR spectrum of the reference compound (Table 1). The active compound showed bright whitish fluorescence on TLC at 366 nm. Its Rf value on TLC was about 0.4 with CHCl3–MeOH–H2O (6 : 4 : 1), and about 0.5 with EtOAc–isopropylalcohol–NH4OH (3 : 4 : 3). Isolation of the active compound at the time of activity detection was possible and the active compound isolated in the on-line system yielded sufficient information to allow identification. Only one more purification step was needed for final structure elucidation.

To obtain a larger quantity of the active compound for the final definite identification, 1 g of extract was separated by means of CPC. The active compound could be easily recognized on TLC at 366 nm from the CPC fractions. It was observed in those fractions which were eluted right after changing the eluent from the mobile phase to stationary phase. The fraction containing the compound of interest was further purified by preparative HPLC, followed by purification with an analytical HPLC column and finally a Sephadex
The isolated compound was identified by co-TLC and co-HPLC together with synthetic ungeremine trifluoroacetic acid (TFA) salt. However, the 1H-NMR signals of the compound and reference compound still showed different shifts. As a change in pH is known to result in considerable shifts in the NMR spectra of alkaloids,13,14 the 1H-NMR spectrum of the reference compound was measured at different pH (in D$_2$O+TFA or D$_2$O+NaOH) (Fig. 6). However none of the spectra obtained matched that of the isolated alkaloid in the two positions (H-1, H-8). By means of co-NMR together with the co-added reference compound, however, it was shown that both compounds indeed were identical. Apparently the concentration of the alkaloid greatly influenced the shifts of some signals (Table 1). The signal of H-1 (at δ 7.42 for reference compound at the highest concentration, and appeared downfield of H-8) showed the biggest shift, but the signal of H-11 also showed clear changes. Upon dilution the H-1 peak shifted downfield to δ 7.65. Since H-8 shifted less than H-1 (from δ 7.56 to δ 7.63), H-1 appeared downfield of H-8 upon dilution. However, when the compound was only in D$_2$O the

Table 1. 1H-NMR Shift of Reference Ungeremine and Isolated Sample in Various Concentrations (600 MHz, in D$_2$O with 0.01% TFA)

<table>
<thead>
<tr>
<th>δ (ppm)</th>
<th>H ref. Ungeremine</th>
<th>about 3× diluted</th>
<th>about 5× diluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>9.155 (s)</td>
<td>9.217 (+0.062)$^b$</td>
<td>9.228 (+0.011)</td>
</tr>
<tr>
<td>11</td>
<td>7.773 (s)</td>
<td>7.954 (+0.181)</td>
<td>7.982 (+0.028)</td>
</tr>
<tr>
<td>8</td>
<td>7.555 (s)</td>
<td>7.617 (+0.062)</td>
<td>7.629 (+0.012)</td>
</tr>
<tr>
<td>1</td>
<td>7.415 (s)</td>
<td>7.617 (+0.202)</td>
<td>7.646 (+0.029)</td>
</tr>
<tr>
<td>3</td>
<td>7.301 (s)</td>
<td>7.363 (+0.062)</td>
<td>7.375 (+0.012)</td>
</tr>
<tr>
<td>OCH$_2$O</td>
<td>6.316 (s)</td>
<td>6.320 (+0.004)</td>
<td>6.320 (+0.000)</td>
</tr>
<tr>
<td>5</td>
<td>5.121 (t, J=6.9)</td>
<td>5.165 (+0.044)</td>
<td>5.172 (+0.007)</td>
</tr>
<tr>
<td>4</td>
<td>3.675 (t, J=6.9)</td>
<td>3.717 (+0.042)</td>
<td>3.724 (+0.007)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>δ (ppm)</th>
<th>H The compound isolated</th>
<th>about 10× diluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>9.189 (s)</td>
<td>9.237 (+0.048)</td>
</tr>
<tr>
<td>11</td>
<td>7.884 (s)</td>
<td>8.009 (+0.125)</td>
</tr>
<tr>
<td>8</td>
<td>7.593 (s)</td>
<td>7.638 (+0.045)</td>
</tr>
<tr>
<td>1</td>
<td>7.537 (s)</td>
<td>7.677 (+0.140)</td>
</tr>
<tr>
<td>3</td>
<td>7.339 (s)</td>
<td>7.385 (+0.046)</td>
</tr>
<tr>
<td>OCH$_2$O</td>
<td>6.319 (s)</td>
<td>6.322 (+0.003)</td>
</tr>
<tr>
<td>5</td>
<td>5.147 (t, J=7.0)</td>
<td>5.180 (+0.033)</td>
</tr>
<tr>
<td>4</td>
<td>3.699 (t, J=7.1)</td>
<td>3.731 (+0.032)</td>
</tr>
</tbody>
</table>

$^a$ Synthetic ungeremine TFA salt.  $^b$ + indicates downfield shift.
signal exchange was not noticed as in D₂O+TFA. We have no clear explanation for the effect of the concentration or the shifts for now. Lack of material made further studies impossible.

The IC₅₀ value measured for ungeremine for AChE inhibition was 0.35 µm, which is about 6—10 times stronger than galanthamine.

Ungernine was first isolated from Ungernia minor,¹⁵ Ungernia spiralis¹⁶ and later from Zephryanthus flava,¹⁷ Crinum asiaticum,¹⁸ Crinum augustum,¹⁹ Pancratium maritimum,²⁰ and Hippeastrum solandriflorum.²¹ It was reported to have growth inhibitory and cytotoxic effect²² and hypotensive properties.²³ The structure–activity relationships concerning its anticancer activity have also been studied.²⁴ Synthesis of ungeremine was also successfully achieved.²⁵,²⁶ However, its AChE inhibitory activity has not been reported before, and ungeremine was isolated from N. bowdenii for the first time.

It seems worthwhile to further study the antiacetylcholinesterase activity of ungeremine and potential structure–activity relationships with related compounds.

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