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RESEARCH ARTICLE

Alkaloids from *Narcissus poeticus* cv. Pink Parasol of various structural types and their biological activity

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Abstract Fifteen Amaryllidaceae alkaloids (**1**–**15**) of various structural types were isolated by standard chromatographic methods from fresh bulbs of *Narcissus poeticus* cv. Pink Parasol. The chemical structures were elucidated by MS, and 1D and 2D NMR spectroscopic analyses, and by comparison with literature data. Narcipavline (**5**) and narcikachnine (**6**) are reported here for the first time. In their structure are combined two basic structural types of Amaryllidaceae alkaloids (galanthamine- and galanthindole-structural types), which represent a new structural type of these compounds. Alkaloids isolated in sufficient amounts were evaluated for their human erythrocytic acetylcholinesterase, and human serum butyrylcholinesterase (HuBuChE) inhibition activity using Ellman's method. *Z*-Gly-Pro-*p*-nitroanilide was used as substrate in the prolyl oligopeptidase (POP) assay. Untested alkaloids were also screened for their cytotoxic activity against a small panel of human cancer cells, which spanned cell lines from different tissue types. In parallel, MRC-5 human fibroblasts were employed to determine overall toxicity against noncancerous cells. Some compounds were evaluated for their antiprotozoal activity. The newly isolated alkaloid narcipavline (**5**) showed interesting HuBuChE inhibition activity ($IC_{50} = 24.4 \pm 1.2$ μM), and norlycoramine (**11**) demonstrated promising POP inhibition ($IC_{50} = 0.21 \pm 0.01$ mM).

Keywords *Narcissus poeticus* cv. Pink Parasol · Amaryllidaceae · Alzheimer's disease · Cytotoxicity · Antiplasmodial activity

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Introduction

Plants of the Amaryllidaceae family, particularly those the *Narcissus* genus have a quite notable place in the history of traditional and Western medicine (Pettit et al. 1984) and are known to contain a specific type of compounds, namely Amaryllidaceae alkaloids, which are of great interest due to their wide range of biological activities, including antiviral, antimalarial, anticancer and anticholinesterasic (Bastida et al. 2011). The alkaloids from their extracts have been an attractive object of phytochemical investigation for nearly 200 years. Over the last three decades many Amaryllidaceae alkaloids have been isolated, screened for various

biological activities, and synthesized by different research groups.

The genus *Narcissus* comprises approximately 80–100 wild species, and is mainly distributed in South-western Europe and North Africa, with some populations in the Balkans, Italy and France. Most *Narcissus* species can hybridize, and hybridization has become very popular with a large number of cultivars having been developed for ornamental purposes, with over 27,000 names of *Narcissus* cultivars now registered in the International Daffodil Register (Kington 2008). The Amaryllidaceous cultivars have advantages for commercial alkaloid production; they are available in large quantities, but only a few studies on alkaloid profile and content in ornamental *Narcissus* cultivars have been published. So far, more than 100 alkaloids of various structural types have been isolated from this genus.

Our unpublished phytochemical study of the alkaloid extracts of various *Narcissus* cultivars previously demonstrated the potential of these plants for commercial isolation of various types of Amaryllidaceae alkaloids. GC–MS analysis of an alkaloid extract of *N. poeticus* cv. Pink Parasol showed a wide range of alkaloids belonging to various structural types. Some of them could not be identified, and it was assumed that these unidentified compounds could be new substances. Moreover, the alkaloid extract showed interesting butyrylcholinesterase inhibition activity ($IC_{50} = 3.3 \pm 0.5$ μM).

Materials and methods

Plant material

The fresh bulbs of *Narcissus poeticus* cv. Pink Parasol were obtained from the herbal dealer Lukon Glads (Sadská, Czech Republic). Botanical identification was performed by one of the co-authors (prof. Lubomír Opletal). A voucher specimen is deposited in the Herbarium of the Faculty of Pharmacy in Hradec Králové under number: CUFPH-16130/AL-298.

Extraction and isolation of alkaloids

Thin layer chromatography (TLC) was carried out on Merck precoated silica gel 60 F_{254} plates, and neutral Al_2O_3 (ACROSS) was used for column chromatography (CC). Silica gel (Kieselgel—mesh 0.15/0.30) was used for all vacuum liquid chromatography (VLC) procedures. For TLC, the development solvents used were mixtures of acetone (Me_2CO), cyclohexane (cHx), toluene (To), chloroform ($CHCl_3$), ethyl acetate (EtOAc), diethylamine (Et_2NH) and aqueous ammonia solution (NH_3) (Penta, Ing. Švec, Praha, Czech Republic). Compounds on the plate were observed under UV light (254 and 366 nm) and visualized by spraying with Dragendorff's reagent.

Fresh bulbs (18.5 kg) were minced and exhaustively extracted with ethanol (EtOH) (96%, v/v, 3×) by boiling for 30 min under reflux; the combined extract was filtered and evaporated to dryness under reduced pressure. The crude extract was acidified to pH 1.5 with 2% hydrochloric acid (HCl; 200 mL), filtered, the filtrate defatted with diethyl ether (Et_2O; 3×), alkalized to pH 9.5 with a 10% solution of Na_2CO_3 and exhaustively extracted with chloroform (4 × 250 mL). The organic layer was evaporated to give 18 g of fluid residue. The extract was acidified to pH 1.5 with 100 mL 2% HCl and defatted again with Et_2O (3 × 200 mL), alkalized to pH 9.5 with a 10% solution of Na_2CO_3 and exhaustively extracted with chloroform (5 × 200 mL). The obtained extract (13 g), which was Dragendorff positive, was further fractionated by CC on Al_2O_3 (840 g), eluting with light petrol gradually enriched with $CHCl_3$ (20:80–10:90), and then $CHCl_3$ enriched with EtOH (99:1–50:50). Fractions of 200 mL were collected and monitored by TLC, yielding 180 fractions, which were combined into 7 fractions, and analyzed by GC–MS.

Preparative TLC (cHx:To:Et_2NH, 24:24:2, 2×) of fraction **I** (296 mg) gave compound **1** (53 mg) and compound **2** (2 mg); both compounds were recrystallized from EtOH. Preparative TLC of fraction I led to sub-fraction Ia, which was separated (To:Et_2NH, 9:1) to give compound **3** (19 mg). Fraction **II** (1.2 g) was chromatographed by preparative TLC (To:Et_2NH, 9:1) to give sub-fractions IIa and IIb. Sub-fraction IIa was further chromatographed by preparative TLC (To:EtOAc:Et_2NH, 50:20:3) to give compound **4** (93 mg). Compounds **5** (7.5 mg) and **6** (5 mg) were obtained from sub-fraction IIb; both compounds were recrystallized from an ethanol and chloroform mixture. The remaining parts of fraction II were chromatographed by VLC to give compound **7** (2 mg) and compound **8** (3 mg); both compounds were recrystallized from EtOH. Preparative TLC of fraction **III** (2 g) (To:Et_2NH, 95:5; 1×) gave compound **9** (13 mg). Fraction **IV** (1.2 g) was treated by preparative TLC and 6 zones were isolated. Sub-fraction IVf was subsequently chromatographed to give compound **10** (85 mg), which was recrystallized from EtOH. VLC of fraction **V** (210 mg) led to the separation of 7 sub-fractions, Va–Vg. Sub-fraction Vc was chromatographed (EtOAc:MeOH, 9:1; 2×) to yield compound **11** (10 mg) and compound **12** (1 mg). Sub-fraction Vb gave two compounds **13** (1 mg) and **14** (2 mg). Fraction **VI** (1.7 g) was treated by preparative TLC (cHx:Me_2CO:NH_3, 20:70:1); 5 zones were isolated. Recrystallization of sub-fraction VIe gave compound **15** (180 mg). Fraction **VII** (2.6 g) was not treated further because of its content of already isolated compounds.

Structural identification of isolated compounds

NMR spectra were recorded for $CDCl_3$ and CD_3OD solutions at ambient temperature on a VNMR S500 NMR (Varian) spectrometer operating at 500 MHz for ^1H and 125 MHz for ^{13}C. Chemical shifts were recorded as δ values in parts per million (ppm), and were indirectly referenced to tetramethylsilane (TMS) via the solvent signal (7.26 ppm for ^1H and 77.0 ppm for ^{13}C for $CDCl_3$, and 3.30 ppm for ^1H and 49.0 ppm for ^{13}C for CD_3OD). Coupling constants (*J*) are given in Hz. For unambiguous assignment of ^1H and ^{13}C signals 2D NMR spectra (COSY, gHSQC, gHMBC and NOESY) were measured using standard parameter settings and pulse programs delivered by the producer of the spectrometer. ESI-HRMS were obtained with a Waters Synapt G7-Si with a hybrid mass analyzer quadrupole-time-of-flight (Q-TOF), coupled to a Waters Acquity I-Class UHPLC system. The EI-MS were obtained on an Agilent 7890A GC 5975 inert MSD operating in EI mode at 70 eV (Agilent Technologies, Santa Clara, CA, USA). A DB-5 column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies, USA) was used. The temperature program was: 100–180 °C at 15 °C/min, 1 min hold at 180 °C, and 180–300 °C at 5 °C/min and 5 min hold at 300 °C; detection range *m/z* 40–600. The injector temperature was 280 °C. The flow-rate of carrier gas (helium) was 0.8 mL/min. A split ratio of 1:15 was used. Optical rotations were measured with a P3000 polarimeter (A. Krüss Optronic) in chloroform.

Preparation of enzymes for human acetylcholinesterase and human butyrylcholinesterase assay

Enzymes were prepared from freshly drawn blood (taken from healthy volunteers), to which 2 mL 3.4% sodium citrate (w/v) per 18 mL blood was added, according to Steck and Kant (1974) with slight modification. Plasma human serum butyrylcholinesterase (HuBuChE) was removed from the whole blood by centrifugation at 4000 rpm in a Boeco U-32R centrifuge fitted with a Hettich 1611 rotor. Red blood cells were transferred to 50 mL tubes and washed 3 times with 5 mM phosphate buffer (pH 7.4) containing 150 mM sodium chloride (centrifugation under same conditions). The washed erythrocytes were stirred with 5 mM phosphate buffer (pH 7.4) for 10 min to ensure lysis. The lysed cells were dispensed for subsequent measurement. Activity of the enzyme preparations was measured immediately after preparation and adjusted with 5 mM phosphate buffer (pH 7.4) to reach activity of blank sample A = 0.08–0.15 for AChE and A = 0.15–0.20 for BuChE.

Human acetylcholinesterase and human butyrylcholinesterase assay

Human erythrocytic acetylcholinesterase (HuAChE) and HuBuChE activities were determined using a modified method of Ellman (Ellman et al. 1961) with ATChI and BuTChI as substrates, respectively. Briefly, 8.3 μL of either blood cell lysate or plasma dilutions (at least 6 different concentrations), 283 μL of 5 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and 8.3 μL of the sample dilution in dimethyl sulfoxide (DMSO) (40, 10, 4, 1, 0.4 and 0 mM) were added to the semi-micro cuvette. The reaction was initiated by addition of 33.3 μL 10 mM substrate (ATChI or BuTChI). The final proportion of DTNB and substrate was 1:1. The increase of absorbance (ΔA) at 436 nm for AChE and 412 nm for BuChE was measured for 1 min at 37 °C using a spectrophotometer (SynergyTM HT Multi-Detection Microplate Reader). Each measurement was repeated 6 times for every concentration of enzyme preparation. The % inhibition was calculated according to the formula: $\%I = 100 - \left(100 \times \frac{\Delta A_{Bl}}{\Delta A_{Sa}}\right)$, where ΔA_{Bl} is the increase of absorbance of the blank sample and ΔA_{Sa} is the increase of absorbance of the measured sample. Inhibition potency of the tested compounds was expressed as IC_{50} value (concentration of inhibitor, which causes 50% cholinesterase inhibition).

Prolyl oligopeptidase assay

Prolyl oligopeptidase (POP; EC 3.4.21.26) was dissolved in phosphate buffered saline (PBS; 0.01 M Na/K phosphate buffer, pH 7.4, containing 137 mM NaCl and 2.7 mM KCl); the specific activity of the enzyme was 0.2 U/mL. The assay was performed in standard polystyrene 96-well microplates with a flat and clear bottom. Stock solutions of tested compounds were prepared in dimethyl sulfoxide (DMSO; 10 mM). Dilutions (10^{-3} to 10^{-7} M) were prepared from the stock solution with deionized H_2O; the control was performed with the same DMSO concentration. POP substrate, (*Z*)-Gly-Pro-*p*-nitroanilide, was dissolved in 50% 1,4-dioxane (5 mM). For each reaction, PBS (170 μL), tested compound (5 μL), and POP (5 μL) were incubated for 5 min at 37 °C. Then, substrate (20 μL) was added and the microplate was incubated for 30 min at 37 °C. The formation of *p*-nitroanilide, directly proportional to the POP activity, was measured spectrophotometrically at 405 nm using a microplate ELISA reader (Multi-mode microplate reader Synergy 2, BioTek Instruments Inc., Vermont, USA). Each measurement was repeated 4 times for every concentration of enzyme preparation. Inhibition potency of tested compounds was expressed as IC_{50} value (concentration of inhibitor which

causes 50% POP inhibition, three independent measurements).

In vitro cytotoxicity study

Cell culture and culture conditions

Selected human tumor and non-tumor cell lines Jurkat (acute T cell leukemia), MOLT-4 (acute lymphoblastic leukemia), A549 (lung carcinoma), HT-29 (colorectal adenocarcinoma), PANC-1 (pancreas epithelioid carcinoma), A2780 (ovarian carcinoma), HeLa (cervix adenocarcinoma), MCF-7 (breast adenocarcinoma), SAOS-2 (osteosarcoma) and MRC-5 (normal lung fibroblasts) were purchased from either ATCC (Manassas, USA) or Sigma–Aldrich (St. Louis, USA) and cultured according to the provider's culture method guidelines. All cell lines were maintained at 37 °C in a humidified 5% carbon dioxide and 95% air incubator. Cells in the maximum range of either 10 passages for primary cell line (MRC-5), or in the maximum range of 20 passages for cancer cell lines (Jurkat, MOLT-4, A549, HT-29, PANC-1, A2780, HeLa, MCF-7 and SAOS-2) and in an exponential growth phase were used for this study.

Cell treatment

All the alkaloids evaluated and doxorubicin, used as positive control, were dissolved in dimethyl sulfoxide—DMSO (Sigma-Aldrich, St. Louis, USA) to prepare stock solutions with a concentration 10–50 mM based on their solubility. Stock solutions were freshly prepared before use in the experiments. For the experiments, the stock solutions were diluted with the appropriate cultivation media to create final concentrations (10 μM for a single-dose alkaloids cytotoxicity screen and 1 μM for doxorubicin used as a reference compound) making sure that the concentration of DMSO was $< 0.1\%$ to avoid toxic effects on the cells. Control cells were sham-treated with a DMSO vehicle only (0.1%; control).

WST-1 cytotoxicity assay The WST-1 (Roche, Mannheim, Germany) reagent was used to determine the cytostatic effect of the tested compounds. WST-1 is designed for the spectrophotometric quantification of cell proliferation, growth, viability and chemosensitivity in cell populations using a 96-well-plate format (Sigma, St.Louis, MO, USA). The principle of WST-1 is based on photometric detection of the reduction of tetrazolium salt to a coloured formazan product. The cells were seeded at a previously established optimal density (30000 Jurkat, 25000 MOLT-4, 500 A549, 1500 HT-29, 2000 PANC-1, 5000 A2780, 500 HeLa, 1500 MCF-7, 2000 SAOS-2 and 2000 MRC-5 cells/well) in 100 μL of culture medium, and adherent cells were allowed to reattach overnight. Thereafter, the cells were treated with 100 μL of corresponding alkaloids or doxorubicin stock solutions to obtain the desired concentrations and incubated in 5% CO_2 at 37 °C. WST-1 reagent diluted 4-fold with PBS (50 μL) was added 48 h after treatment. Absorbance was measured after 3 h incubation with WST-1 at 440 nm. The measurements were performed in a Tecan Infinite M200 spectrometer (Tecan Group, Männedorf, Switzerland). All experiments were performed at least three times with triplicate measurements at each drug concentration per experiment. The viability was quantified as described in Havelek et al. (2012) according to the following formula: (%) viability $= (A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}}) \times 100$, where A is the absorbance of the employed WST-1 formazan measured at 440 nm. The viability of the treated cells was normalized to the viability of cells treated with 0.1% DMSO (Sigma-Aldrich, St. Louis, MO, USA) as a vehicle control.

Statistical analysis The descriptive statistics of the results were calculated and the charts made in either Microsoft Office Excel 2010 (Microsoft, Redmond, WA, USA) or GraphPad Prism 5 biostatistics (GraphPad Software, La Jolla, CA, USA). In this study, all of the values were expressed as arithmetic means with SD of triplicates ($n = 3$), unless otherwise noted. The significant differences between the groups were analyzed using the Student's t test and a P value ≤ 0.05 was considered statistically significant.

Anti-plasmodial assay

Collection of sporozoites

Females of *Anopheles stephensi* mosquitoes were infected by feeding on infected mice using standard methods of mosquito infection (Ploemen et al. 2009). Salivary glands were collected in DMEM (Dulbecco's Modified Eagle Medium, Gibco/Invitrogen) and homogenized in a grinder. The homogenate was filtered and centrifuged at 3000 RPM for 5 min. Free sporozoites were then counted in a Neubauer chamber using phase-contrast microscopy.

In vitro activity against P. berghei liver stage

Human hepatoma cell line (Huh-7 cells) was cultured in 1640 RPMI medium (Gibco/Invitrogen) supplemented with 10% v/v fetal calf serum (FCS) (Gibco/Invitrogen), 1% v/v penicillin/streptomycin (Gibco/Invitrogen), 1% v/v nonessential amino acids (Gibco/Invitrogen), 1% v/v glutamine (Gibco/Invitrogen) and 10 mM HEPES, pH 7, (Gibco/Invitrogen) and maintained at 37 °C with 5% CO_2.

Inhibition of *P. berghei* liver-stage infection was determined by measuring the luminescence of Huh-7 cell lysates 48 h after infection with a firefly luciferase-expressing *P. berghei* line, PbGFP-Luccon, as previously described (Ploemen et al. 2009; Prudêncio et al. 2011; Ribeiro et al. 2016). Huh-7 cells (10^4 cells per well) were seeded in a 96-well plate the day before drug treatment and infection. Test compounds were prepared as 10 mM stock solutions in DMSO and diluted with 1640 RPMI medium supplemented with 0.1% v/v gentamicin and 0.32% fungizone to the desired screening concentrations (10, 5, and 1 μM). Each concentration was assayed in triplicate. Medium was replaced by fresh medium containing the appropriate concentration of each compound 1 h prior to cell infection by *P. berghei* sporozoites. Sporozoites (10^4 spz per well) were freshly obtained through disruption of salivary glands of infected mosquitoes (see above). Infected cells in a 96-well plate were centrifuged at 3000 RPM for 5 min and incubated at 37 °C, with 5% CO_2.

The effect of the compounds on the viability of Huh-7 cells was assessed by the AlamarBlue® assay (Invitrogen) following the manufacturer's protocol. Fluorescence intensity was measured by using a Tecan Infinite M200 spectrometer (Tecan Group, Switzerland) at 530 nm excitation wavelength/590 nm emission wavelength. Parasite load was determined 48 h after infection by luminescence measurement using a Luciferase Assay System Kit® (Promega, Netherlands). The 96-well plate was washed out with phosphate buffer and cells were incubated with 75 μL of lysis buffer for 15 min in a shaker at 600 RPM. After the lysis, the 96-well plate was centrifuged for 5 min at 3000 RPM to deposit debris and membranes. Thirty μL of the supernatant was transferred into a white 96-well plate and the luciferase activity was measured by adding 50 μL of Luciferase Assay substrate (1:50 dilution) (Luciferase Assay System Kit®, Promega, Netherlands). Luminescence intensity was measured by using a Tecan Infinite M200 spectrometer (Tecan Group, Switzerland) with a 5 s duration of the light reaction. Statistical analysis was performed by using Tecan I control software (Tecan Group, Switzerland) and Microsoft Office Excel 2010 (Microsoft, USA). Nonlinear regression analysis was employed to fit the normalized results of the dose–response curves.

Results and discussion

Extensive chromatographic purification led to the isolation of two new alkaloids (**5**, **6**), which were isolated along with 13 known alkaloids (Fig. 1). The known alkaloids were identified by comparison with published spectroscopic and physical data as narwedine (**1**; Jegorov et al. 2006), homolycorine (**2**; Huang et al. 2003), masonine (**3**; Pigni et al. 2012), lycoramine (**4**; Chen et al. 2012), 10-*O*-demethylhomolycorine (**7**; Huang et al. 2003), galanthamine (**8**; Chen et al. 2012), seco-isopowellaminone (**9**; Kogure et al. 2011), oduline (**10**; Huang et al. 2003), norlycoramine (**11**; Bastida et al. 2006), haemanthamine (**12**; Bastida et al. 2006), galanthindole (**13**; Unver et al. 2003), incartine (**14**; Berkov et al. 2007), and hippeastrine (**15**; Jeffs et al. 1985).

The isolated alkaloids belong to the homolycorine (**2**, **3**, **7**, **10**, **15**), galanthamine (**1**, **4**, **8**, **11**), haemanthamine (**9**, **12**), galanthindole (**13**), and lycorine (**14**) structural types. The galanthamine and galanthindole structural types of Amaryllidaceae alkaloids are combined in the structure of the newly isolated alkaloids. The structures are similar to the pallidiflorine structural type, in which galanthamine and tazettine structural types are combined. The novel compounds, narcipavline (**5**) and narcikachnine (**6**), were obtained as a light yellow viscous mass. ESI-HRMS of **5** showed a molecular ion peak $[M+H]^+$ at *m/z* 539.2542, corresponding to the formula $C_{33}H_{34}N_2O_5$ (calc. 539.2541). The ESI-HRMS of compound **6** could not be measured due to the instability of the compound. The complex structures of the isolated compounds **5** and **6** were determined by employing 1D and 2D NMR experiments (Table 1). In both cases, a mixture of two stereoisomers was obtained; in a 1:1 ratio for compound **5** and a 1:1.2 ratio for compound **6**. Measurement of the ^{13}C NMR spectra revealed that both compounds are similar to each other and contain 33 carbon atoms. Compound **5** contains 20 carbons in the aromatic and 13 carbons in the aliphatic area. One of them, strongly unshielded, corresponds to the O–CH_2–O group (shifts 102.5 and 102.6 ppm). qHSQC experiment showed that compound **5** further contains two methyl groups, six methylene groups, two methine carbons and one aliphatic quaternary carbon atom. The first methyl carbon (chemical shift 56.7 ppm) corresponds to an OCH_3 group and the second one (36.0 and 35.8, resp.) to an NCH_3 group. Furthermore, three methylene carbons in the aliphatic area are clearly adjacent to an NCH_2 moiety, and a methine carbon (69.9 and 65.9 ppm, resp.) corresponds to an –OCH– group. gCOSY and gHMBC experiments determined the constitution of compound **5**. The NMR spectrum of compound **6** resembles that of compound **5** with 33 carbon atoms detected. However, there are 18 aromatic carbons and 15 aliphatic carbons. 2D NMR experiments led to the conclusion that compound **6** contains in its molecule an *N*-methyl-2,3-dihydroindole substructural fragment instead of the *N*-methylindole moiety in compound **5**. The results of gHMBC experiments allowed to identify three substructural fragments: 4a,5,7,8,9,10,11,12-octahydro-6*H*-benzo[2,3]benzofuro[4,3-*cd*]azepine (fragment A), benzo[*d*][1,3]dioxole (fragment B) and *N*-methylindole (**5**) or *N*-methyl-2,3-dihydroindole (**6**)

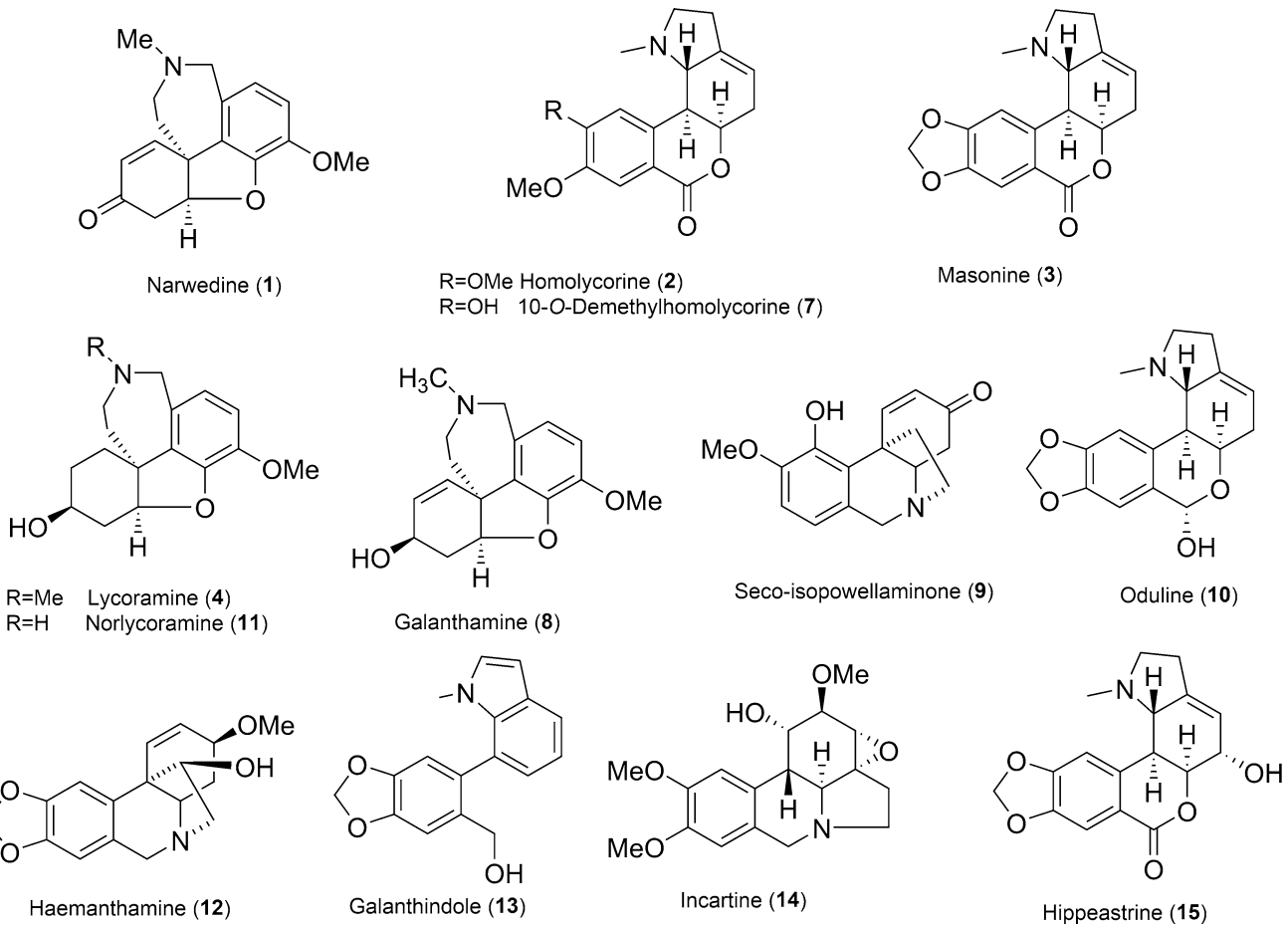

Fig. 1 Structures of isolated alkaloids from *Narcissus poeticus* cv. Pink Parasol

(fragment C), respectively. Based on the correlations obtained from gHMBC experiment (Fig. 2), fragment A is attached to fragment B via methylene group (1′) which connects the position 10 of fragment A with the position 2′ of fragment B. Indole or 2,3-dihydroindole, resp., is bonded to the position 8′ of the fragment B, clearly confirmed by gHMBC experiment revealing the correlation of hydrogen attached to carbon 10′ with carbon 8′ of fragment B. Key gHMBC correlations of compounds **5** and **6** are shown in Fig. 2.

All isolated compounds obtained in sufficient amounts were assayed for their human erythrocytic acetylcholinesterase (HuAChE), HuBuChE, and POP inhibition activities; biological activities which are connected with the treatment of Alzheimer's disease (AD). AChE inhibition is the current approach for AD treatment. Galanthamine hydrobromide and Huperzine A were used as positive controls in the HuAChE and HuBuChE assays, *Z*-Pro-prolinal and berberine were used as positive controls in the POP assay. The results, expressed as IC_{50} values, are summarized in Table 2. In the HuAChE assay, with the exception of galanthamine, which is a known AChE inhibitor and already used in the therapy of AD under the commercial name Reminyl© (galanthamine hydrobromide), only homolycorine showed moderate inhibition activity, with an IC_{50} value of 64 ± 4 μM. However, not only AChE participates in the cholinergic regulation of the central nervous system in humans, but also another enzyme, butyrylcholinesterase (BuChE), which is able to hydrolyze acetylcholine (Ach; Nicolet et al. 2003), and may be a significant contributor to the observed loss of ACh in AD (Walsh et al. 2011). The most potent HuBuChE inhibition activity has been demonstrated by the newly isolated and described alkaloid narcipavline (IC_{50} = 24.4 ± 1.2 μM). Unfortunately, the other new alkaloid narcikachnine, which differs only in the presence of a double bond in position C′13–C′14 in narcipavline instead of a single bond in narcikachnine (Fig. 2), could not be tested, due to the low amount of compound obtained upon isolation. This would have shown whether the presence of a double bond is crucial for BuChE inhibition activity.

POP is a cytosolic serine peptidase that cleaves peptide bonds at the carboxyl end of proline, and is widely

Table 1 ^{1}H- and ^{13}C-NMR data of new alkaloids **5** and **6** isolated from *Narcissus poeticus* cv. Pink Parasol

Position	**5** in CD_3OD		**6** in $CDCl_3$	
	^{1}H	^{13}C	^{1}H	^{13}C
1	1.72–1.53 m	25.3 and 25.2	1.88–1.47 m	23.8 and 23.7
2	1.72–1.53 and 1.42–1.23 m	27.9 and 27.8	1.88–1.47 m	27.7
3	4.02–3.93 m	65.9 and 65.9	4.04 bs	65.5 and 65.5
4	2.25–2.15 and 1.91–1.77 m	32.8 and 32.7	2.46 t (J = 14.7 Hz) and 1.88–1.47 m	31.5 and 31.4
4a	4.02–3.93 and 3.87–3.80 m	90.4 and 90.4	4.28–4.25 and 4.14–4.11 m	90.0 and 89.9
5a	–	147.8 and 147.8	–	146.0
6	–	145.3 and 145.1	–	143.9
7	6.66–6.63 m and 6.56 d (J = 8.2 Hz)	112.7 and 112.6	6.62–6.57 m	110.6 and 110.5
8	6.34 d (J = 8.2 Hz) and 6.29 d (J = 8.2 Hz)	122.6 and 122.3	6.51 d (J = 8.3 Hz) and 6.40 d (J = 8.3 Hz)	121.7 and 121.7
8a	–	130.3	–	130.1
8b	–	137.4 and 137.2	–	136.6
9	3.87–3.80, 3.78–3.72 and 3.54–3.45 m	59.3 and 58.7	3.89 d (J = 15.0 Hz) and 3.88 d (J' 15.0 Hz) and 3.65 d (J = 15.0 Hz) and 3.52–3.43 m	58.6 and 58.3
11	2.96–2.87 and 2.71–2.64 m	52.6 and 52.5	3.05–2.85 and 2.83–2.72 m	52.3 and 51.2
12	1.42–1.23 m	33.1 and 32.3	1.88–1.47 and 1.44–1.37 m	32.1 and 31.2
13	3.36–3.24 m and 3.24–	48.0 and 47.9	3.52–3.42 m and 3.39–	46.7 and 46.7
1′	3.11 m	55.4 and 54.2	3.32 m	54.9 and 53.7
2′	–	133.1 and 132.9	–	131.9
3′	7.03 and 7.01 s	110.1 and 109.9	7.06 s and 7.04–6.99 m	108.7 and 108.5
3′a	–	148.8 and 148.8	–	146.9 and 146.9
5′	6.01–5.99 m	102.6 and 102.5	5.98–5.96 m	100.9 and 100.9
6′a	–	147.5 and 147.3	–	145.9 and 145.7
7′	6.76 and 6.74 s	111.9 and 111.8	6.75 and 6.72 s	110.4 and 110.3
8′	–	135.0 and 134.7	–	133.6 and 133.4
9′	–	126.0 and 125.8	–	122.8 and 122.8
10′	6.83 dd (J = 7.4 Hz, J = 1.0 Hz) and 6.66–6.63 m	124.9	6.82 d (J = 7.1 Hz and 6.68–6.62 m	130.4 and 130.2
11′	7.03–6.96 m and 6.89 t (J = 7.4 Hz)	119.7 and 119.6	6.66 t (J = 7.1 Hz) and 6.62–6.57 m	118.0 and 117.9
12′	7.48 dd (J = 7.7 Hz, J = 1.0 Hz) and 7.46 dd (J = 7.7 Hz, J = 1.0 Hz)	121.0 and 120.9	7.04–6.99 m	123.2
12′a	–	131.2 and 131.1	–	131.0
13′	6.43 d (J = 2.9 Hz) and 6.39 d (J = 2.9 Hz)	101.8 and 101.8	3.03–2.85 and 2.83–2.72 m	28.6 and 28.6
14′	7.03–6.96 and 6.94–6.92 m	131.8 and 131.7	3.31–3.21 m and 3.17 dd (J = 9.1 Hz, J = 9.1 Hz) and 3.08 dd (J = 9.1 Hz, J = 9.1 Hz)	57.1 and 56.9
15′a	–	135.9 and 135.8	–	150.5 and 150.4
6-OCH_3	3.80 and 3.77 s	56.7 and 56.7	3.85 s	55.9 and 55.9
15′-NCH_3	3.27 and 3.16 s	36.0 and 35.8	2.23 and 2.18 s	38.7 and 38.7

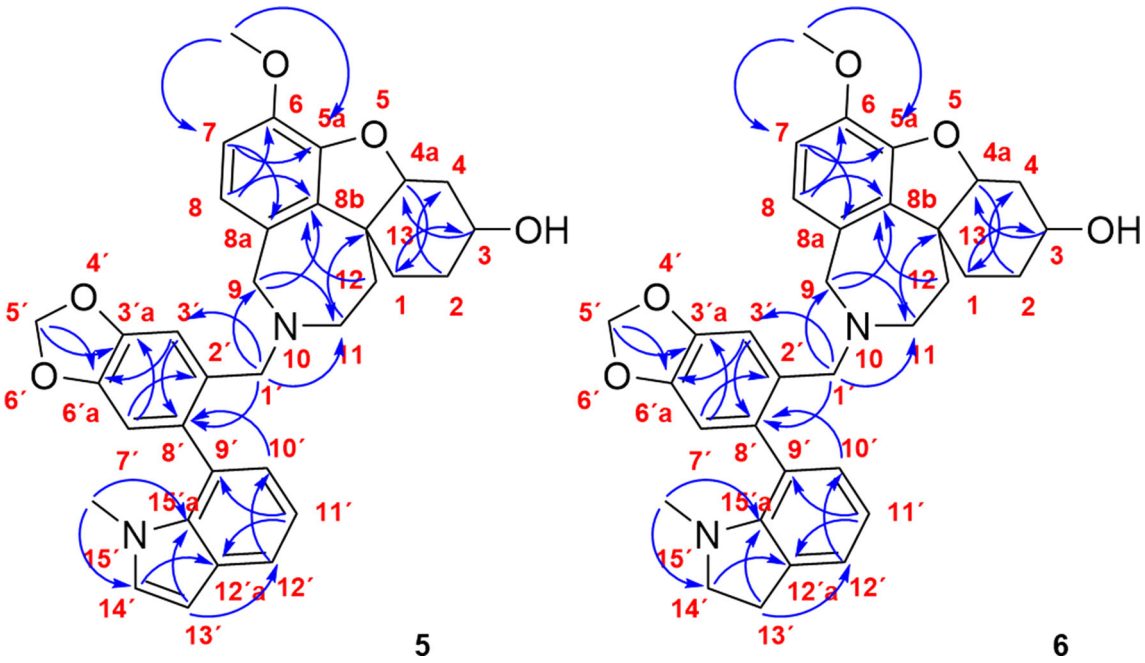

Fig. 2 Key gHMBC correlations of new compounds **5** and **6** isolated from *Narcissus poeticus* cv. Pink Parasol

Table 2 HuAChE, HuBuChE, and POP inhibitory activity of the tested Amaryllidaceae alkaloids isolated from *Narcissus poeticus* cv.

Compound	HuAChE IC^{a}_{50} (μM)	HuBuChE IC^{a}_{50} (μM)	POP IC^{a}_{50} (μM)
Narwedine (**1**)	281 ± 33	> 500	> 1000
Homolycorine (**2**)	64 ± 4	151 ± 19	173 ± 41
Masonine (**3**)	304 ± 34	229 ± 24	314 ± 34
Lycoramine (**4**)	456 ± 57	> 500	> 500
Narcipavline (**5**)	208 ± 37	24.4 ± 1.2	nm[b]
Narcikachnine (**6**)	nm[b]	nm[b]	nm[b]
10-*O*-demethyhomolycorine (**7**)	nm[b]	nm[b]	nm[b]
Galanthamine (**8**)	1.7 ± 0.1	42.3 ± 1.3	> 1000
Seco-isopowellaminone(**9**)	293 ± 33	> 500	> 1000
Oduline (**10**)	> 500	> 500	252 ± 17
Norlycoramine (**11**)	> 500	> 500	209 ± 14
Haemanthamine (**12**)	> 500	> 500	> 500
Galanthindole (**13**)	nm[b]	nm[b]	nm[b]
Incartine (**14**)	208 ± 14	> 500	> 1000
Hippeastrine (**15**)	> 500	> 500	> 1000
Galanthamine[c]	1.7 ± 0.1	42.3 ± 1.3	> 1000
Huperzine A[c]	0.033 ± 0.001	> 500	–
Berberine[c]	–	–	142 ± 21
Z-Pro-prolinal	–	–	2.75×10^{-3}

Pink Parasol expressed as IC_{50}

[a]Results are the mean values ± standard deviations of three independent replications

[b]nm: not measured due to limited material

[c]Standard

distributed in the organs of the body, including the brain (García-Horsman et al. 2007; Polgár 2002). In recent studies, some POP inhibitors have been found to be efficacious antidementia drugs (Orhan 2012). Thus, POP inhibition can represent an important supporting approach in AD treatment, and therefore, we tested the isolated

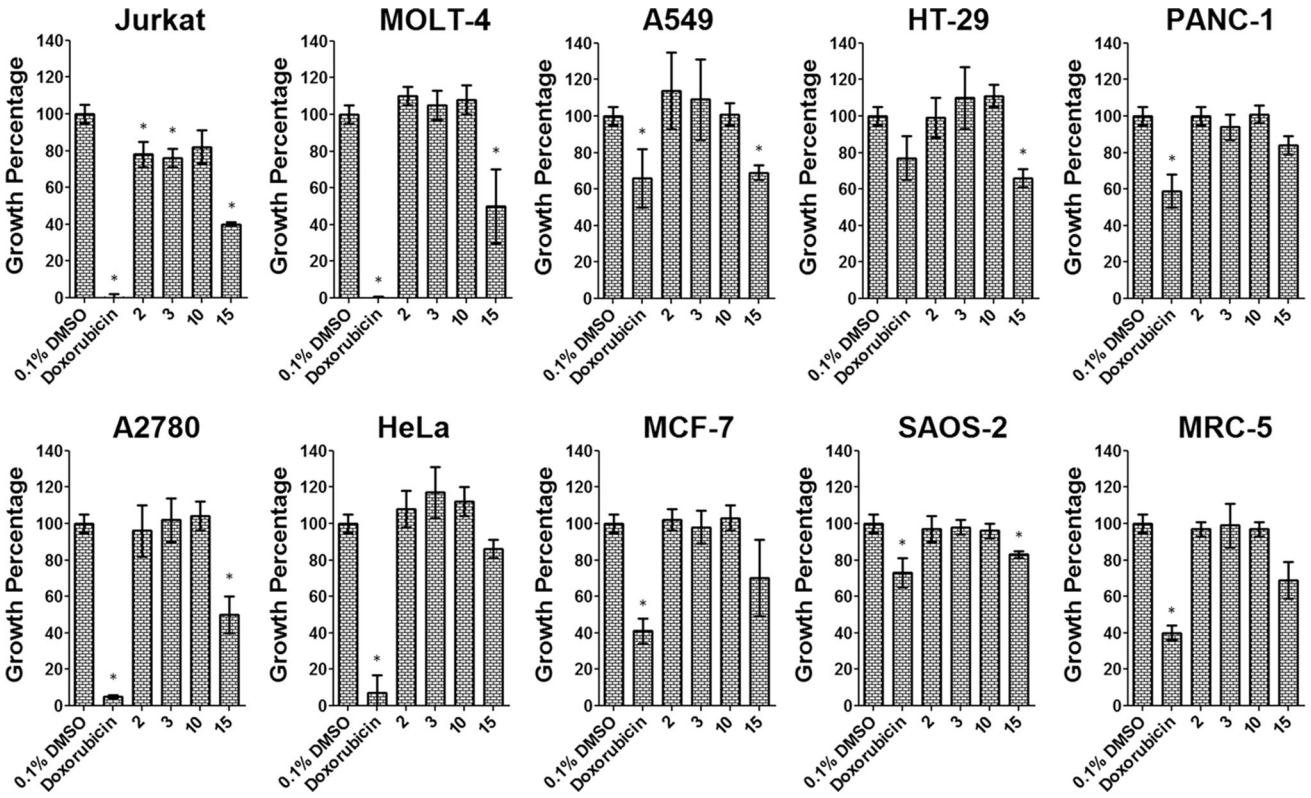

Fig. 3 Cytotoxic activity of **2**, **3**, **10** and **15** following a single-dose exposure at a concentration of 10 μM. Cell growth was measured by using a WST-1 assay 48 h after treatment. The results are expressed as the mean values ± SD of at least three independent experiments (n = 3). Cells treated with 1 μM doxorubicin were used as positive control.* $P \leq 0.05$ versus 0.1% DMSO sham-treated control

compounds for their ability to inhibit POP. The most interesting inhibition activity was demonstrated by the homolycorine-type alkaloid homolycorine (**2**), with an IC_{50} value of 173 ± 41 μM. Obtained inhibition activity is comparable with POP inhibition activity of isoquinoline alkaloid berberine (IC_{50} = 142 ± 21 μM), which has been used as a standard. Further promising POP inhibitions have also been shown by the galanthamine-type alkaloid norlycoramine (IC_{50} = 209 ± 14 μM) and other homolycorine-type alkaloid oduline (IC_{50} = 252 ± 17 μM; Table 2). Some of the Amaryllidaceae alkaloids have been previously tested for their POP inhibition activity; the best result has been shown by the lycorine type alkaloid 9-*O*-demethylgalanthine (IC_{50} = 150 ± 20 μM) isolated from *Zephyranthes robusta* (Šafratová et al. 2014).

Alkaloids of the homolycorine-type (**2**, **3**, **10** and **15**) that were isolated in sufficient amounts were also tested for their cytotoxic activity against a small panel of human cancer cells (Jurkat, MOLT-4, A549, HT-29, PANC-1, A2780, HeLa, MCF-7 and SAOS-2), spannding cell lines from different tissue types. In parallel, normal MRC-5 human fibroblasts were employed to determine the compounds' overall toxicity against non-cancer cells. The cytotoxic activity of these alkaloids was evaluated using the WST-1 metabolic activity assay. In this screening process, the alkaloids were tested for growth-inhibitory activity in all 10 cell lines at a single dose of 10 μM (Fig. 3). For each alkaloid tested, the sensitivity in an individual cell line and the mean growth percent (GP) value was calculated as an average of 10 cell lines proliferation in percent. The threshold GP value for this screen was < 50% (50% tumor growth inhibition), indicating good activity at 10 μM. In this work, only compound **15** was found to inhibit Jurkat, MOLT-4, and A2780 cancer cell growth with a score ≤ 50% at 10 μM concentration (Table 3). Notably, the antiproliferative activity of compound **15** at 10 μM leads to a 40% growth inhibition in Jurkat, and a 50% growth inhibition in both MOLT-4 and A2780 cells. Also, incubation of A549, HT-29 and SAOS-2 cells with compound **15** led to a statistically significant ($P \leq 0.05$) reduction of cell proliferation. Contrary thereto, compounds **2**, **3** and **10** showed poor anticancer activity against cancer cells of different histotypes evaluated in this study. Compared with the activity of the chemotherapy agent approved for cancer therapy, doxorubicin, none of the evaluated alkaloids exhibited a stronger overall activity

Table 3 Sensitivity to the antiproliferative actions of **2**, **3**, **10** and **15** following a single-dose exposure at a concentration of 10 μM

Compound	Mean GP[a]	Range of GP[b]	Most sensitive cell lines	% inhibition
2	100	78–114	Jurkat, A2780, MRC-5	78*, 96, 97
3	101	76–117	Jurkat, PANC-1, SAOS-2	76*, 94, 98
10	101	82–112	Jurkat, SAOS-2, MRC-5	82, 96, 97
15	67	40–86	Jurkat, MOLT-4, A2780	40*, 50*, 50*

* Significantly different to 0.1% DMSO sham-treated control ($P \leq 0.05$)

[a]Mean growth percent (GP) value was calculated for each compound as an average of 10 cell lines proliferation in percent

[b]Range of growth percentage, as well as the three most sensitive cell lines with growth percentage values are indicated for each compound

against any of the 10 cell lines than doxorubicin (mean GP 37%). It is worth-mentioning that compound **15** showed a much lower antiproliferative activity than the other alkaloid representatives of the Amaryllidaceae family previously evaluated by our group. Among these, lycorine and **12** showed very potent cytotoxic activity in the micromolar range against the cancer cells tested (Doskocil et al. 2015; Havelek et al. 2014).

Some of the Amaryllidaceae alkaloids are also of particular interest because of their potential antimalarial activity as lycorine, haemanthamine, haemanthidine and crinamine have benn shown to possess activity against the blood stages of several strains of *Plasmodium falciparum* (Campbell et al. 2000; Sener et al. 2003; Cedrón et al. 2010). However, following the bite of an infected mosquito, and prior to infecting red blood cells, *Plasmodium* parasites undergo an obligatory developmental phase in their mammalian host's liver (Ploemen et al. 2009). This stage of the parasite's development is clinically silent and therefore regarded as an ideal point for either vaccine strategies or prophylactic intervention (Prudêncio et al. 2006; Matuschewski 2007) Thus, we also evaluated the activity of compounds **1**–**4**, **8**, **10**, **12** and **15** against the hepatic stage of *P. berghei*. However, these compounds did not display any discernible activity ($IC_{50} > 100$ μM) against *P. berghei* liver stages.

In conclusion, two new, along with 13 known Amaryllidaceae alkaloids were isolated from fresh bulbs of *Narcissus poeticus* cv. Pink Parasol. New described Amaryllidaceae alkaloids narcipavline and narcikachnine demonstrate new structural type of these compounds. The plant cultivar employed in this study is a rich source of diverse Amaryllidaceae alkaloids for pharmaceutical research, especially those of the homolycorine type. Compounds isolated in sufficient amounts were assayed for their biological activities connected with Alzheimer's and oncological diseases. Additionally, the anti-Plasmodial activity of the alkaloids has been evaluated. Some alkaloids were isolated in amounts that will allow detailed study of their mechanism of action, as well as the preparation of new derivatives for biological assays and for structure–activity relationship studies.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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