Pyrrolizidine alkaloids from *Solenanthus lanatus* DC. with acetylcholinesterase inhibitory activity

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1. Introduction

*Solenanthus lanatus* DC. (Boraginaceae) is a small herbaceous plant characterised by tubular flowers and alternate lanceolate leaves, entirely covered with white silver hairs, particularly dense in the inflorescence. The species is endemic to the Morocco–Algeria area (Beniston & Beniston 1984). Its leaves are used locally in traditional medicine for the treatment of eye and skin diseases, burns and wounds, while other species in the genus have been reported to possess an analgesic effect (Ranjbar 2009).

ABSTRACT

The whole plant ethanolic extract of *Solenanthus lanatus* was used for the isolation of acetylcholinesterase inhibitors. A new pyrrolizidine alkaloid, 7-O-angeloylechinatine N-oxide, 1, was isolated together with three known compounds of the same class (3′-O-acetylheliosupine N-oxide, 2, heliosupine N-oxide, 3, and heliosupine, 4), by bioassay-guided approach. Their structures were elucidated by spectroscopic methods. All the isolated compounds showed inhibition activity against the AChE, with IC\textsubscript{50} 0.53–0.60 mM.

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Previous phytochemical investigations on some Boraginaceae genera, including *Solenanthus* have led to isolation and identification of pyrrolizidine alkaloids (PAs) as main components (El-Shazly & Wink 2014). To our knowledge, there are no previous phytochemical studies describing the secondary metabolites of *S. lanatus*. After a preliminary biochemical screening which showed an anti-acetylcholinesterase (AChE) activity for the ethanolic extract of the plant, we decided to pursue bioassay-guided isolation of its main active components. Herein, we report the isolation, structure elucidation and AChE inhibitory activities of four PAs from the whole plant of *S. lanatus*, including the new compound 7-angeloylechinatine N-oxide (1), together with three known compounds: 3'-acetylheliosupine N-oxide (2) (Constantinidis et al. 1993), heliosupine N-oxide (3) (Asibal et al. 1989), and heliosupine (4) (Asibal et al. 1989; El-Shazly et al. 1996) (Figure 1). The crude extract and fractions from *S. lanatus* were also tested.

**Figure 1.** Structures of the alkaloids isolated from *Solenanthus lanatus* (1–4).
2. Results and discussion

2.1. Identification of isolated compounds

The phytochemical study of *S. lanatus* whole plant led to the isolation and characterisation of four PAs, 1 (7-angeloylchinatine N-oxide) was a new natural product (see Figure 1), while 2 (3′-acetylheliosupine N-oxide), 3 (heliosupine N-oxide) and 4 (heliosupine) were previously isolated from the aerial parts of *Cynoglossum creticum* and *Heliotropium hirsutissimum*, but never been reported before in the genus *Solenanthus*. The spectroscopic data of compounds 2, 3 and 4 are in agreement with those reported in the literature (Asibal et al. 1989; Constantinidis et al. 1993; El-Shazly et al. 1996).

7-angeloylchinatine N-oxide (1) was obtained as a brown gum. Its molecular formula was established as C_{20}H_{31}NO_{7} (six degrees of instauration) from HRESIMS for the peak at *m/z* 398.2221 [M + H]+ (Calcd for C_{20}H_{32}NO_{7}, 398.2179). The 13C NMR spectrum showed 20 carbon atoms. The multiplicity assignments were determined using DEPT experiments which displayed five methyl, four methylene, six methine and five quaternary carbon atoms. Both the 1H and 13C NMR spectra showed the presence of an angelic acid and a (−)-viridifloric acid moieties and a base structure characterised by a 1,2-unsaturated necine in the form of N-oxide. The nature of angeloyl moiety was determined based on the chemical shift of H-3″ at δ 6.26 (in the case of a tigloyl moiety, H-3″ would resonate at lower field). A careful comparison of its MS and NMR spectra with the literature data allowed to assign to 1 a heliotridine structure (El-Shazly et al. 1996). In fact, in the NOESY spectrum no NOE was observed between the H-7 and H-8, confirming the trans configuration of the protons (Roeder et al. 1991). Also, the chemical shift values of C-6 (δ 31.3), C-7 (δ 74.7) and C-8 (δ 96.3), together with H-7 (δ 5.28) and H2–9 (δ 4.93 and 5.04), showed that 1 is a heliotridine diester, while the HMBC spectrum, evidencing couplings between C-1′ and H-9b, indicated that heliotridine is esterified at C-7 by the angelic acid and at C-9 by the (−)-viridifloric acid. Based on the reported evidences, the structure of 1 was therefore assigned as 7-angeloylchinatine N-oxide (Figure 1).

The obtained results could be interesting in the light of a potential application in pest control. It is well known that the two main classes of cholinesterase inhibiting pesticides are the organophosphates (OPs) and the carbamates (CMs) (Thacker 2002; Costa et al. 2008). However, the use of these compounds, like other chemical pesticides, is in discussion, due to effects on the environment and resistance. Resistance is an emerging phenomenon and new insecticides, able to replace the previous ones, are urgently needed (Karaagac 2011). Novel pesticides, in order to be suitable, should be low-cost, eco-friendly, from renewable raw material, nontoxic to non-target organisms, of rapid degradation and non-accumulating in the environment and most of the research now is dedicated to plant natural compounds (Nicoletti et al. 2010). Some chemicals, derived from plant metabolites, such as the chlorinated derivatives of nicotine, can also affect the cholinesterase enzyme (Thacker 2002). Also, PAs are explored in such direction (Macel et al. 2005; Narberhaus et al. 2005; Thoden & Boppré 2010). Therefore, plants containing PAs could be considered a promising source for models in developing new insecticides, suitable for integrated pest management (Lima et al. 2011).

2.2. Acetylcholinesterase inhibition

The bioassay-guided fractionation of the ethanolic extract of whole plant of *S. lanatus* led to the isolation of four compounds, 1–4. The isolated compounds were tested on TLC for their
potential inhibition activity against AChE. All compounds were active at 15 μg, galanthamine used as positive control was active in the test at 1 μg. The ethanolic extract and fractions C, D, E were also active at 15 μg and showed positive spots with a violet background on TLC. Other fractions (A and B) were inactive at the same level. The spectrophotometric assay of AChE inhibitory activity of isolated compounds (Table 1), showed a lower activity (IC₅₀ values ranging of 0.53–0.60 mM) than the positive control galanthamine (IC₅₀ value of 0.00081 mM). The ethanolic extract and fractions from S. lanatus were active in the following order of potency: fraction C > fraction D > ethanolic extract > fraction E. Moreover, several alkaloids, including pyrrolizidine class, have been reported to interact with nicotinic and muscarinic acetylcholine receptors (Green et al. 2013; Tasso et al. 2013; Wink 2013; Xu et al. 2013). The isolated compounds probably could bind to these kinds of receptors, potentially having synergistic effects acting both as an AChE inhibitor and acetylcholine receptors agonist.

3. Experimental procedure

3.1. General experimental procedure

Optical rotations were measured on a Jasco DIP-370 digital polarimeter (Jasco, Easton, MD, USA). NMR spectra were recorded on Varian Mercury 300 MHz (Varian, Palo Alto, CA, USA) and/or Bruker AVANCE 400 NMR spectrometer (Bruker, Karlsruhe, Germany), in CDCl₃ or CD₃OD (TMS as internal standard).

MS spectra were performed on a Q-Tof MICRO spectrometer (Waters, Manchester, UK) equipped with an ESI source, which was operated in the positive ion mode. Instrument Configuration: Collision Gas 1.0; Inlet Gas 1.0; Backing Pirani 2.69e0; Analyser Penning 4.07e-9; Tof Penning 1.72e-7; Cone(L/hr) 20; Desolvation (L/hr) 500; Instrument Parameters: Polarity ES+; Capillary (V) 3000.0; Sample Cone (V) 45.0; Extraction Cone (V) 2.0; Desolvation Temp (°C) 200.0; Source Temp (°C) 80.0; Syringe Rate (ul) 10.0; Ion Energy (V) 1.0; Collision Energy 3.0; Low Mass Resolution 5.0; High Mass Resolution 5.0. Start Mass 100.0; End Mass 1000.0;

| Compounds | IC₅₀ (mM) ± SEMb |
|-----------|------------------|
| EtOH extract | 467.71 ± 6.77 μg/mL A |
| Fraction A | >500 μg/mL |
| Fraction B | ND |
| Fraction C | 198.34 ± 2.12 μg/mL B |
| Fraction D | 424.21 ± 2.32 μg/mL C |
| Fraction E | 468.93 ± 6.01 μg/mL A |
| (1) | 0.597 ± 0.007D |
| (2) | 0.537 ± 0.005E |
| (3) | 0.602 ± 0.002F |
| (4) | 0.570 ± 0.003F |
| Galanthaminec | 0.00081 ± 0.00001G |

aConcentrations of compounds that caused 50% enzyme activity loss.
bStandard error of the mean of three assays.
cGalanthamine was used as positive control.
ND is not determined due to the interaction of fraction B with reagents.
A–G: Differences within column (samples not connected by the same letter are statistically different at P < 0.05).
Concentration range of reaction for ethanolic extract and fractions (A–E): from 100 to 500 µg/mL
Concentration range of reaction for compounds (1–4): from 50 to 250 µg/mL
Concentration range of reaction for galanthamine: from 0.25 to 04 μM
R² ranged from 0.89 to 0.99
Capillary (V) 3000.0; Sample Cone (V) 45.0; Extraction Cone (V) 2.0; Desolvation Temp (°C) 200.0; Source Temp (°C) 80.0.

Data were analysed using the MassLynx software developed by Waters.

AChE inhibitory assays were performed using a spectrophotometer 2120 UV OPTIZEN (Mecasys Co., Ltd, Korea).

3.2. Chemicals and reagents

Acetylcholinesterase from electric eel (EC 3.1.1.7, type V-S, lyophilised powder, 827 U/mg solid, 1256 U/mg protein), acetylthiocholine iodide (ATChI), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 2-naphthyl acetate, fast blue B salt, galanthamine hydrobromide, tris and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA). Four different buffer systems were used (buffer A: 50 mM tris-HCl, pH 7.8, buffer B: 50 mM tris-HCl, pH 7.8 containing 0.1% BSA, buffer C: 50 mM tris-HCl, pH 8 containing 0.1% BSA and buffer D: 50 mM tris-HCl, pH 8 containing 0.1 M NaCl and 0.02 M MgCl₂·6H₂O). All remaining chemicals used were of analytical grade and were purchased from Sigma.

3.3. Plant material

S. lanatus DC. was collected during the flowering period (February 2014) in the commune of Tamzoura, Ain Temouchent, Algeria, and identified by one of the authors (Professor A. MAROUF). Voucher specimens were deposited in the collections of the lab of the first author (OUE2014-C1). The whole plant was dried in a well-ventilated room at a temperature of 30 °C and stored in the dark until use.

3.4. Extraction and isolation

Dried powdered whole plant of S. lanatus (2 kg) was extracted with EtOH at room temperature for 72 h. The extract was filtered and evaporated at 40 °C under reduced pressure to yield 109.6 g. This crude extract was shaken for 30 min with 2 M HCl (500 mL) and filtered. The insoluble residue was collected, dried and extracted with acetone (fraction A, 19.0 g). The neutral material was removed from the filtrate with n-hexane (fraction B, 0.2 g). Both fractions A and B tested negative with the Dragendorff’s reagent, indicating an absence of alkaloids. The acidified solution was then basified with 25% NH₄OH up to pH 10 and extracted with CHCl₃ (3 × 1000 mL) to give a chloroformic extract (fraction C), containing an alkaloid mixture (2.2 g). To obtain alkaloids in reduced form, the aqueous phase was re-acidified with HCl up to pH 2, reduced with Zn dust overnight, filtered and made alkaline with 25% NH₄OH to pH 10. The alkaline solution was then extracted with CHCl₃ (3 × 1000 mL) to give a second chloroformic extract (fraction D), containing reduced alkaloids (9.5 g). The remaining alkaline solution constituted the fraction E. All fractions were submitted to TLC AChE assay (see below). The fractions C and D were found to be active in the assay. The fraction C was subjected to silica gel (40–63 μm, Merck) column chromatography (2.5 × 55 cm), eluted with gradient of CH₂Cl₂-MeOH (100:0–0:100) to give twelve fractions (C1–C12). The fraction C9 (303 mg) was found to be more active after TLC AChE assay and was further purified by preparative TLC silica gel 60 (F₂₅₄ glass plate 1 mm thickness, Merck), eluted with CH₂Cl₂-MeOH-NH₄OH (85:15:02) to give compounds 1 (11 mg), 2 (48 mg) and 3 (53 mg). Fraction D was purified on silica gel...
(40–63 μm, Merck) CC (3 × 65 cm), eluted with gradient of CH₂Cl₂-MeOH (100:0–0:100) to obtain nine fractions (D1–D9). Fraction D5 (3.742 g) was active after TLC AChE assay and was separated over preparative TLC silica gel 60 (F₂₅₄ glass plate 1 mm thickness, Merck), eluted with CH₂Cl₂-MeOH-NH₄OH (64:16:02) to obtain compound 4 (66 mg).

3.4.1. 7-angeloylchalinatine N-oxide (1)
Brown gum; [α]D²² = +3.5 (c 0.50, MeOH). HRESIMS m/z: 398.2221 [M + H]+ (Calcd 398.2179 for C₂₀H₃₂N₂O₇). ESI-MS m/z: 398 (80), 301 (36), 239 (39), 229 (25), 185 (43), 118 (100). ¹H NMR, ¹³C NMR, COSY (400 MHz, CD₃OD) δ 0.94 (3H, d, J = 6.5 Hz, H-6′), 0.96 (3H, d, J = 6.5 Hz, H-7′), 1.17 (3H, d, J = 6.8 Hz, H-4′), 1.84 (1H, m, H-5′), 1.93 (1H, s, H-5″), 2.03 (1H, d, J = 7.6 Hz, H-4″), 2.38 (1H, m, H-6b), 2.47 (1H, m, H-6a), 3.82 (1H, m, H-5b), 3.94 (1H, br. d, J = 15.8 Hz, H-3b), 4.68 (1H, s, H-8), 4.70 (1H, br. d, J = 15.8 Hz, H-3a), 4.93 (1H, d, J = 14.0 Hz, H-9b), 5.04 (1H, d, J = 14.0 Hz, H-9a), 5.28 (1H, bs, H-7), 6.04 (1H, s, H-2), 6.26 (1H, q, J = 7.6 Hz, H-3″). ¹³C NMR (100 MHz, CD₃OD): 16.5 (C-4″), 17.1 (C-7), 17.4 (C-6″), 17.7 (C-4″), 20.5 (C-5″), 31.3 (C-6), 34.2 (C-5′), 61.6 (C-9), 68.9 (C-5), 70.5 (C-3′), 74.7 (C-7), 78.3 (C-3), 83.2 (C-2′), 96.3 (C-8), 124.9 (C-2), 128.2 (C-2″), 133.0 (C-1), 141.4 (C-3″), 167.6 (C-1″), 173.1 (C-1″).

3.5. Acetylcholinesterase inhibitory assay
3.5.1. TLC bioautography assay
The TLC AChE assay was conducted as described by (Mroczek 2009). AChE (500 U) was dissolved in buffer A to make 1000 U/mL stock solution and further diluted with buffer B to obtain 3 U/mL enzyme. The stock solution was kept at −20 °C. The ethanolic extract of S. lanatus, fractions and isolated compounds were applied at level of 15 μg on TLC silica gel 60 F₂₅₄ aluminium sheets. Galanthamine (1 μg) was included on the plates as a positive control. After migration of the samples in a suitable mobile phase but containing additionally 1.5 mg mL⁻¹ of 2-naphthyl acetate, the TLC plates were air-dried. The plates were then sprayed with 3 U mL⁻¹ of enzyme solution, thoroughly dried again and incubated for 10 min at 37 °C in humid atmosphere. After, the plates were pulverised by the fast blue B salt solution (1.25 mg mL⁻¹ in water). The violet background was revealed after 1 min.

Other extracts, obtained from the same plant by maceration at room temperature with different solvents (hexane, dichloromethane, ethyl acetate; 5 g for 30 mL, 24 h, repeated three times) or by reflux with water (5 g for 50 mL, 30 min, repeated three times) were tested in the same way and proved to be inactive.

3.5.2. Spectrophotometric assay
AChE was assayed according to the principle of hydrolysis of ATChI by monitoring the formation of 5-thio-2-nitrobenzoate spectrometrically at 412 nm (Ellman et al. 1961) and was conducted as in Ingkaninan et al. (2000) with slight modification. The reaction mixture contained 295 μL of buffer C, 100 μL of 0.226 U/mL AChE in buffer C, 5 μL of S. lanatus ethanolic extract or fractions or isolated compounds or galanthamine (as positive control) in methanol at five different concentrations or methanol (as negative control) and 500 μL of 3 mM DTNB in buffer D, which were mixed and incubated for 15 min at 37 °C. The reaction was then initiated in cuvettes by addition of 100 μL of 15 mM ATChI in water and after, cuvettes were shaken for 5 s and the changes in absorbance were measured at 412 nm every 20 s for 4 min with Optizen 2120 UV spectrophotometer. All the reactions were performed in triplicate and the initial
rates were measured as the rate of change in absorbance/time (Purich 2010). Percentage of inhibition of AChE was determined by using the formula \( 1 - \left( \frac{R_{\text{sample}}}{R_{\text{control}}} \right) \times 100 \), where \( R_{\text{sample}} \) is the rate of reaction of sample and \( R_{\text{control}} \) is that of the negative control (Nair et al. 2011). The AChE inhibitory activity was expressed in terms of the IC\(_{50}\) value (sample concentration required to inhibit the hydrolysis of the substrate by 50%).

3.6. Statistical analysis of the data

All the tests were carried out in triplicate. Results are expressed as mean ± standard error mean (SEM). IC\(_{50}\) values were calculated from the linear regression line obtained by plotting the percentage of inhibition of AChE versus the inhibitor concentrations. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Student-Newman–Keuls’ post hoc test for multiple comparisons. A value of \( p < 0.05 \) was considered to indicate statistical significance.

4. Conclusion

In summary, four PAs were isolated from \( S. \) lanatus and their structures were characterised by spectroscopic means (\(^1\)H and \(^{13}\)C NMR and mass spectrometry experiments). One of them, \( 1 \), is a new PA, while the other three are known compounds, whose occurrences have been reported in the present study for the first time in the genus \( Solenanthus \). The results showed that all the isolated compounds possess AChE inhibitory activity.

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