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Hedgehog antagonists cyclopamine and dihydroveratramine can be mistaken for each other in *Veratrum album*

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ABSTRACT

A toxic plant, *Veratrum album* (ssp. *viriscens*), was found to have an inhibitory effect on Hedgehog (Hh), a developmental signaling pathway that has been shown to be active during development, in adult stem cells and in numerous human tumors. Based on earlier studies it was believed that the known Hh inhibitor cyclopamine was present in *V. album* (ssp. *viriscens*). Here we show that instead of cyclopamine, dihydroveratramine (DHV) was found in *V. album* (ssp. *viriscens*). These compounds are easily mistaken for each other, as both substances share the same molecular weight, and the same main MS/MS fragments. DHV was found to be a less potent Hh inhibitor compared to cyclopamine. This is the first reported occurrence of DVH in nature.

1. Introduction

The Hedgehog signaling pathway (Hh) plays a crucial role in development, adult stem cells, and has been associated with tumor progression and possibly cancer stem cells (CSCs) [1,2]. Several Hh antagonists have shown potential as anti-tumor agents *in vitro* and *in vivo* [3–5]. The first identified Hh pathway antagonist was the natural occurring cyclopamine (11-deoxyjervine, Fig. 1(I)), present in *Veratrum californicum* Durand [6]. A substantial body of evidence links cyclopamine to the direct inhibition of the transmembrane receptor Smoothened (SMO) [7,8]. The species similarity of the American *V. californicum* and the European *Veratrum album* encouraged us to investigate whether *V. album* could contain cyclopamine related substances with SMO inhibitory activity. Although *V. album* has been used sporadically for various medicinal purposes including e.g. by ancient ethnic groups in the north of Norway [9], the plant has been shown to be highly toxic [10,11]. Previously, cyclopamine has been reported to be present in *V. album* L. var. *glandiflorum album* (*V. glandiflorum* Loesen. fil.) [12]. In contrast, we here demonstrate the presence of dihydroveratramine (DHV, Fig. 1(III)), instead of cyclopamine, in another subspecies of *V. album* (ssp. *virescens Gaudin*). Interestingly, DHV has not previously been reported as a natural product. DHV is easily mistaken for cyclopamine, as both molecules share the same molecular formula, similar retention times in gradient mode reverse phase (RP) separations, and produce similar mass fragmentation spectra. Similar to cyclopamine, DHV was also found to be an Hh signal antagonist, although less potent.

2. Materials and methods

2.1. Chemicals and solutions

Cyclopamine and veratramine were purchased from Toronto Research Chemicals (North York, Ontario, Canada). All solvents were of HPLC grade, and were tested to be ineffective on Hh pathway signaling at relevant concentrations. Veratramine was used as an additional external standard for determining DHV concentrations in solutions used to test bioactivity.

2.2. Sample extraction and isolation

Veratrum album (ssp. *virescens*) was collected in July 2009 in the Systematic Garden, Natural History Museum, Oslo, Norway.

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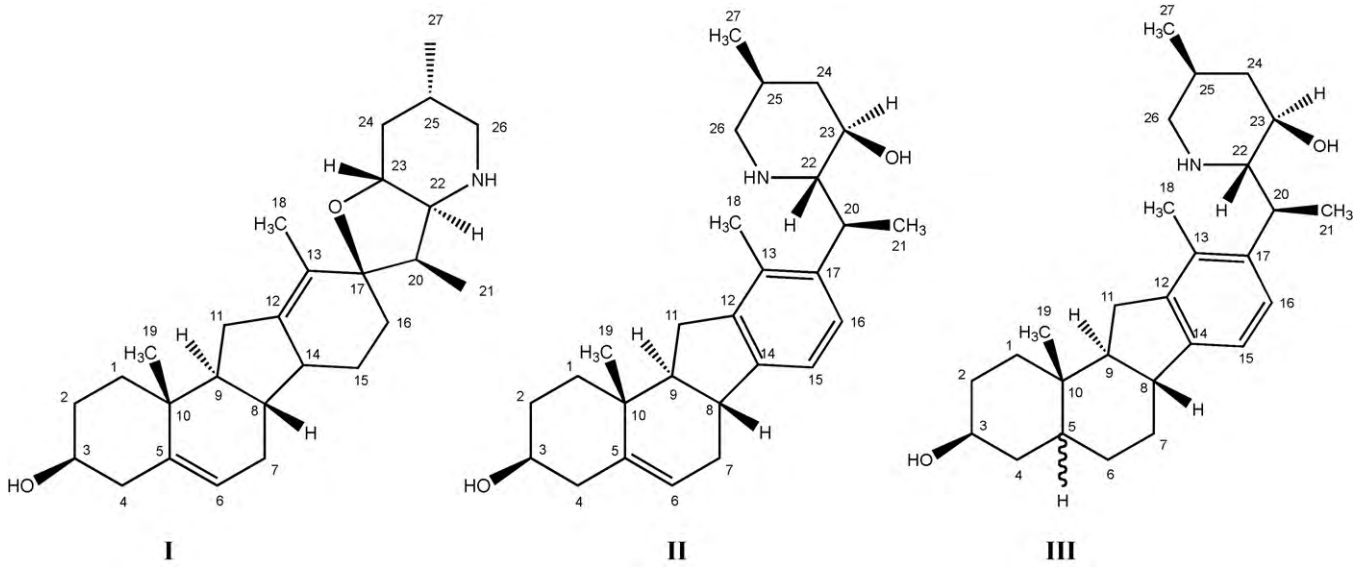

Fig. 1. (I) Cyclopamine, (II) veratramine; and (III) dihydroveratramine (DHV).

The plant was identified by botanist Dr. Hanne Hegre Grundt. A voucher specimen was deposited at the Department of Chemistry, Oslo, Norway (Ø128-1). Roots from *V. album* (ssp. *viriscens*) (Supplementary material, SM-PIC) were air-dried for 5 days and 340 g dry roots were powdered and wet with 6% NH_4OH (*aq*) (1 mL/g root). The roots were extracted under shaking with 4 × 0.5 mL benzene at room temperature (3 h per extraction). The brown/yellow extract (2.8 g) was concentrated at reduced pressure, and redissolved in dichloromethane/isopropanol (85:15, v/v). The solution was subjected to normal phase (NP)-flash chromatography, with 250 mL (50 mL fractions) 85:15 (fractions 1–5), 80:20 (6–10), 75:25 (11–15), 70:30 (16–20) and 60:40 (21–25) dichloromethane/isopropanol (v/v). The fractions were subject to ESI–MS. Fraction 10 containing the highest amount of mass 412.3 was subjected to reversed phase (RP)-flash chromatography, with 250 mL (50 mL fractions) of 10, 20, 30, 40, 50, 75, 100% ACN (*aq*, v/v), respectively. The first 40% ACN fraction was high in 412.3 Da concentration (>98%), and the solvent was evaporated under reduced pressure to dryness. A total yield of 5 mg DHV was obtained. A Supelco (Bellefonte, PA, USA) Versapak 40 mm × 150 mm (20–45 μm spherical silica) cartridge was used for NP preparative flash chromatography, and a Versapak C_{18} cartridge (40 mm × 150 mm) for RP preparative flash chromatography.

In a preliminary experiment, a sample collected in June 2008 was analyzed by LC–MS.

2.3. Analysis

UV spectra were measured on a Cary 100 Bio instrument (Varian, Palo Alto, CA, USA). HPLC analytical separations were performed with an 1100 series Agilent (Santa Clara, CA, USA) pump and Zorbax C_{18} capillary column (0.3 mm × 15 cm) that was coupled directly to a mass spectrometer. All analytical HPLC separations were performed using a solvent gradient from 95% solvent A ACN–H_2O–FA (5:94.9:0.1, v/v/v) to 95% solvent B ACN–H_2O–FA (95:4.9:0.1, v/v/v) in 40 min. MS^n data was recorded on a Esquire IT–MS (Bruker, Bremen, Germany) and high resolution MS and MS/MS data was recorded on TOF-LCT (Waters, Milford, MA, USA) and Orbitrap (Thermo, Waltham, MA, USA) spectrometers. DHV had an *m*/*z* measured to 412.320 $[M+H]^+$, with MS/MS fragments 394.3 and 297.2 (>99%).

2.4. Structural elucidation (NMR) and quantum chemical calculations

See Supplementary material section (SM-NMR-E and SM-QQ).

2.5. Biological assays

Root extracts and the isolated DHV were tested for Hh activity using sonic Hedgehog (Shh) and Smoothened Agonist (SAG) induced Shh-Light-2 cells based on methodology developed by Taipale et al. [7]. The Shh-Light-2 cells were either activated with 50% Shh conditioned medium, or by 200 nM SAG.

3. Results and discussion

3.1. Biological effect of DHV

Shh and SAG induced Shh-Light-2 cells were treated with serial dilutions of a dried benzene extract from the root of *V. album* (ssp. *viriscens*). In Shh activated cells, the root extract had a 50% inhibitory activity at a dilution of about 1:50,000, and a 100% inhibitory activity at a dilution of 1:5000. In contrast, the inhibitory action of the extract in 200 nM SAG activated cells was 50% at a dilution of approximately 1:3000, and 100% at 1:1000.

Isolated DHV blocked Hh signaling with an IC50 of approximately 8 μM, similar to that of veratramine, while cyclopamine had an IC50 of approximately 0.3 μM. SAG induced Hh signaling was blocked by cyclopamine with an IC50 of approximately 2 μM, and DHV had an SAG antagonizing IC50 > 10 μM. 4% (w/w) of the extract was measured to be DHV.

3.2. Liquid chromatography and mass spectrometry

As the presence of cyclopamine in *V. album* was described in the literature, it was assumed that the Hh inhibitory activity found in *V. album* (ssp. *viriscens*) was due to the presence of cyclopamine [12]. Initial MS analysis indeed revealed substantial amounts of a compound with the same molecular weight ($[M+H]^+ = m/z$ 412.320) and the same main MS/MS fragments as cyclopamine (Fig. 2). The lack of several of the cyclopamine fragments in the *V. album* ssp. *viriscens* compound was first believed to be due to performance variations with the MS instrumentation. However, when using a

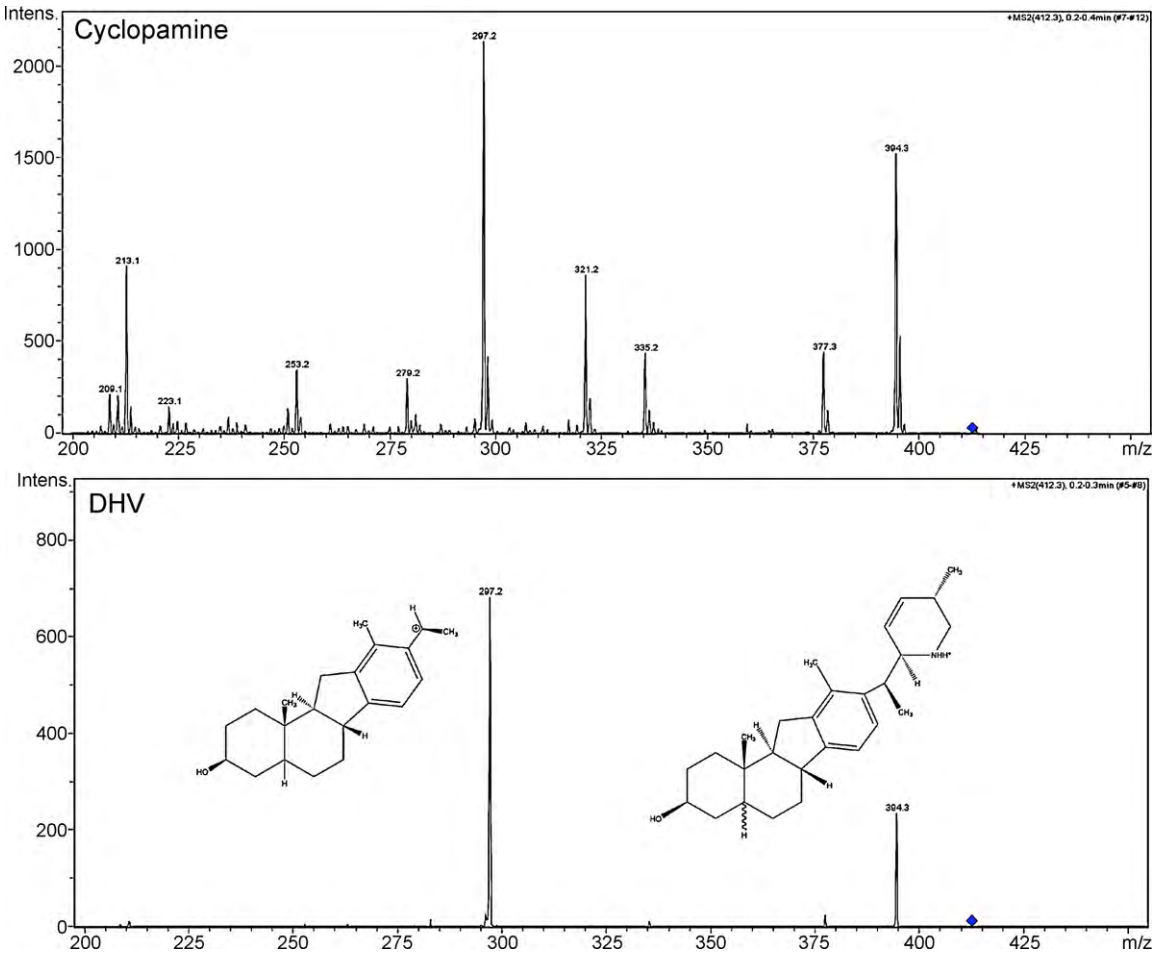

Fig. 2. Ion trap MS/MS spectra of cyclopamine (top) and DHV (bottom). Both compounds share the same major fragments (394.3 and 297.2 Da).

shallow LC gradient, it was evident that the *V. album* ssp. *viriscens* derived compound had a shorter retention time. In confirmation, also LC–MS analysis of plant material spiked with commercially available cyclopamine revealed two distinct peaks. The compound found in *V. album* ssp. *viriscens* was therefore rather believed to be an isomer of cyclopamine (Fig. 3).

Since the RP retention time was slightly shorter, it was likely that an additional hydrophilic functional group was present. A part of the isolated compound was diluted with D_2O, and the deuterated compound had a 1 Da higher mass than deuterated cyclopamine (SM-MS1), implying that the compound had an extra hydroxyl compared to cyclopamine.

3.3. NMR and UV spectroscopy

The presence of an extra hydroxyl group was supported by the ^{13}C APT (attached proton test) NMR data, that showed four non-protonated aromatic carbons and two protonated aromatic carbons (SM-NMR1) suggesting that the substance had no spiro carbon, and hence no ether bridge. Aromaticity was confirmed by UV spectroscopy, which revealed a local wavelength maximum at 267 nm (SM-UV1), implying a homo-annular conjugated system [13]. Since fragmentation beyond an aromatic ring structure is less energetically plausible compared to the saturated ring structure in cyclopamine, this corresponded with the limited MS/MS fragmentation in the isomer compared to cyclopamine.

The finding of an aromatic ring in the *V. album* ssp. *viriscens* derived substance led us to revisit the published ^{13}C NMR data of the aromatic alkaloid veratramine (Fig. 1(II)) [14]. These data are listed to the left in Table 1. If the compound had been a ring-opened isomer of cyclopamine, only four ^{13}C NMR resonances in the olefinic/aromatic region would have been observed. On the other hand, if it was a ring-opened compound (cyclopamine isomer) that had been oxidized/dehydrogenated and formed veratramine, eight olefinic/aromatic resonances should have been present in this region, namely carbon atoms 5, 6, 12–17 (SM-NMR2). The fact that only six aromatic signals were observed in the published ^{13}C-NMR data led us to believe that the compound derived from *V. album* ssp. *viriscens* had a reduced C5–C6 fragment in combination with a ring opening and oxidation leading to an aromatic ring. The combined results from the one dimensional ^{1}H spectrum, ^{13}C APT spectrum, an edited HSQC spectrum, a HMBC spectrum and a NOESY spectrum, all showing 27 carbon atoms, are summarized in Table 1 (Table 1, column 4). The actual spectra as well as a TOCSY spectrum (not discussed) are deposited as supplementary material (SM-ADD, NMR).

The calculated chemical shift value of carbon 21 allowed establishing the *cis* stereo-chemical relationship between H5 atom and the 19-CH_3 group. Carbon 21 in the *trans*-isomer was predicted to have a shift of 12 ppm while the *cis*-isomer would show a predicted shift of 20 ppm. Our experimentally found value of 21.02 ppm strongly indicated a thermodynamically non-favored *cis*-isomer (Fig. 4). A NOESY NMR experiment further confirmed the *cis*-arrangement (Fig. 4). A final verification of the structure was done by a comprehensive interpretation of the HMBC spectrum (Table 1) with the rest of the NMR spectroscopic data. The integrated picture

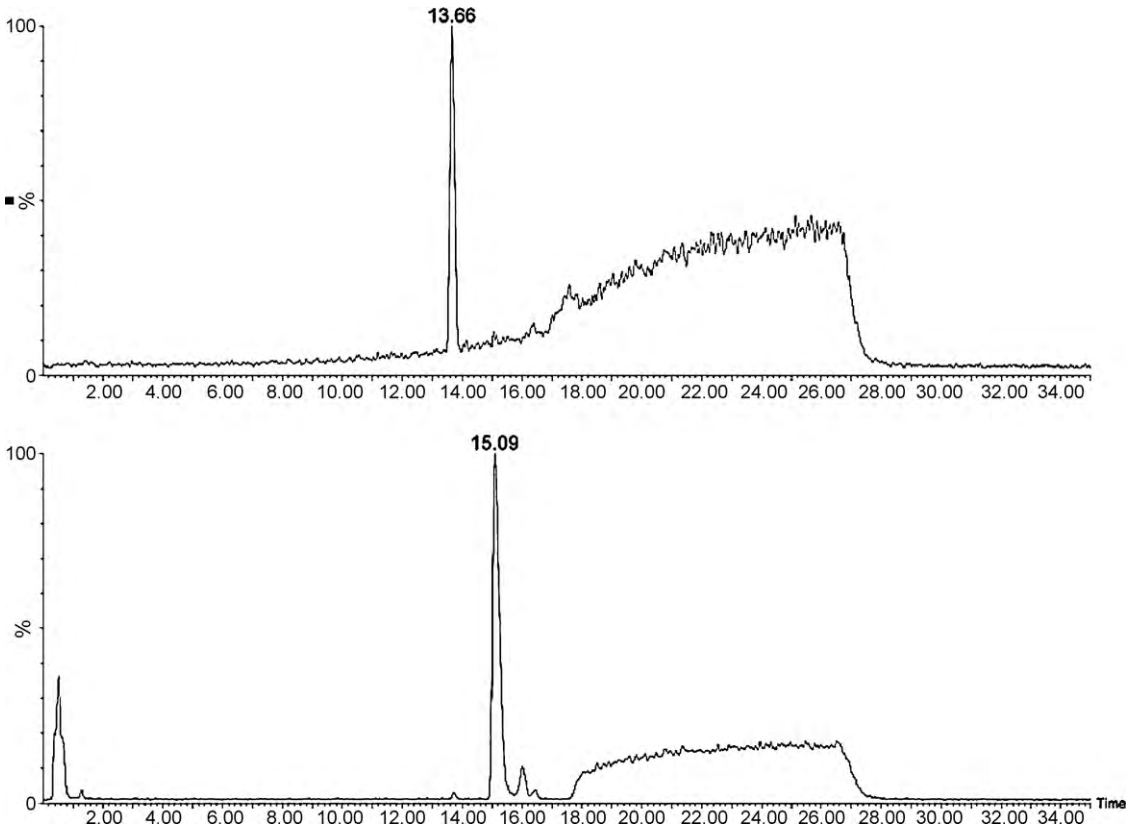

Fig. 3. LC–TOF–MS chromatogram of dihydroveratramine (DHV) (top) and cyclopamine (bottom). See Sections 2.2 and 2.3 for conditions.

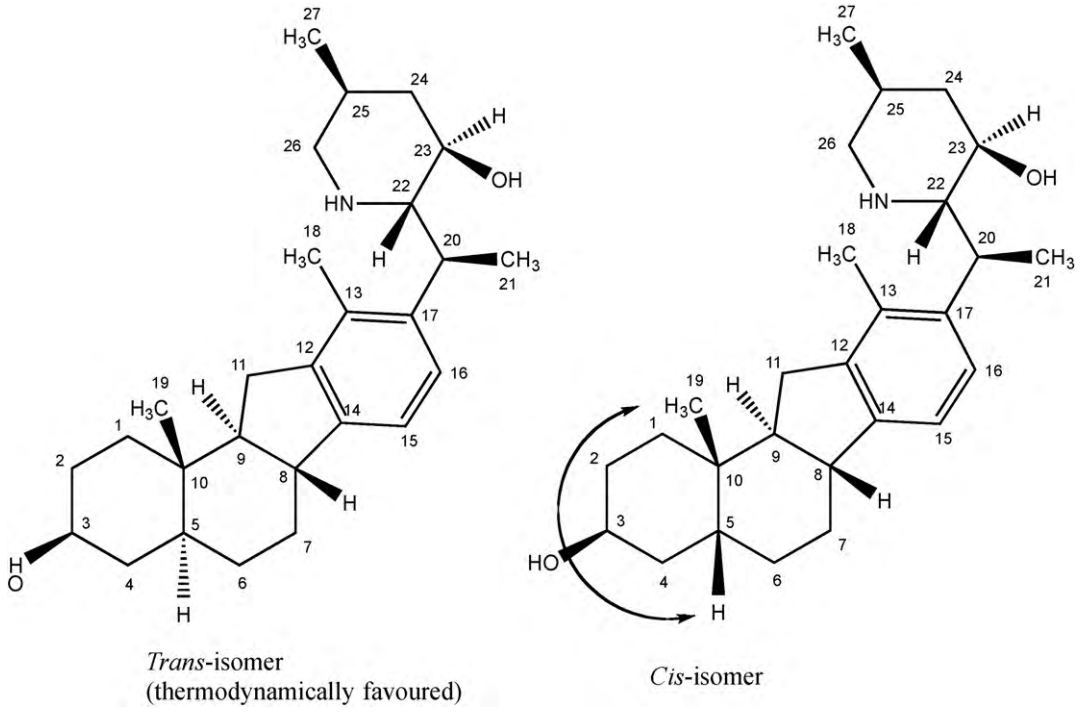

Fig. 4. NOESY correlation indicated between H5 and Me-19 in *cis*-isomer.

Table 1
Predicted and observed NMR shift values of dihydroveratramine.

Position	d_C, veratra-mine (II)[a]	d_C, dihydroveratramine (III) calculated[b]	d_C, dihydro-veratramine	d_H (J in Hz)	HMBC[c]	HMBC[d]	Selected NOESY
1	38.5 CH_2	40.8 CH_2	31.97 CH_2	1.71, dq, 2.71, 13.70 1.46, dd, 4.38, 13.70	2, 9		
2	32.1 CH_2	30.6 CH_2	28.64 CH_2	1.65, dt, 14.58, 2.99 1.57, dt, 12.14, 2.99			
3	71.3 CH	72.3 CH	67.58 CH	4.02, b, 2.49	1, 5		
4	42.9 CH_2	42.9 CH_2	34.60 CH_2	1.94, dq, 13.55, 2.71 1.40, m	1.94–6		(1.94 to) 19
5	142.7 qC	141.1 CH	36.97 CH	1.89 m		3	
6	121.5 CH	121.8 CH_2	29.01 CH_2	2.06, m, 1.39 m	(2.06 to) 5	7	
7	30.8 CH_2	32.5 CH_2	25.54 CH_2	2.10, m, 1.47 m		9	
8	41.5 CH	44.1 CH	45.89 CH	2.83, dd, 12.65, 3.58	14	15	
9	57.5 CH	60.2 CH	48.50 CH	2.23, dt, 12.82, 6.74	8, 11, 18, 19	19	
10	37.2 qC	40.8 qC	36.05 qC			19	
11	30.7 CH_2	32.5 CH_2	31.38 CH_2	2.69, bt, 12.45 2.39, bq, 12.60	2.69–8, 9, 12, 13, 14 2.39–9, 12		
12	143.65 qC[e]	139.6 qC	144.59 qC				
13	133.1 qC[e]	131.0 qC	134.01 qC				
14	143.69 qC[e]	139.8 qC	147.65 qC				
15	119.9 CHe	113.7 CH	121.01 CH	7.02, d, 7.61	18		
16	126.7 CH	129.4 CH	126.11 CH	7.07, d, 7.61	18		
17	141.2 qC	134.8 qC	137.46 qC				
18	16.1 CH_3	19.4 CH_3	16.03 CH_3	2.34, s	12, 13, 15, 17		
19	19.3 CH_3	21.1 CH_3	23.99 CH_3	1.08, s	12, 13, 15, 16, 17		4
20	35.7 CH	46.5 CH	35.69 CH (broad)	3.78, m			18, 21, 22
21	21.1 CH_3	17.0 CH_3	21.02 CH_3	1.44, d, 7.27	13, 16, 17	22	
22	68.3 CH	74.3 CH	67.38 CH	3.07, dd, 4.85, 10.14	17, 20, 21	17, 18, 20, 21	
23	70.7 CH	69.0 CH	68.32 CH	3.47, dt, 4.51, 10.59	20, 22	(3.47 to) 20	
24	45.2 CH_2	46.0 CH_2	42.71 CH_2	2.11, m, 1.27 m	1.27–22, 25, 26, 27		
25	32.5 CH	32.1 CH	28.37 CH	1.86, m			
26	54.6 CH_2	55.5 CH_2	52.16 CH_2	3.12, dq, 12.19, 1.90 2.61, t, 12.56	22, 24, 25, 27		
27	19.0 CH_3	20.5 CH_3	18.35 CH_3	0.98, d, 6.76	24, 25, 26		

[a] Literature values from 10 in CD_3CN.
[b] More calculated values; see SM-MCC.
[c] HMBC correlations from proton(s) stated to the indicated carbon.
[d] HMBC correlations from carbon stated to the indicated hydrogen.
[e] The authors state that these values might be interchanged. Most likely the values for C12 and C13 are intermixed in the reference.

of the HMBC correlations is shown visually in a ChemDraw picture (SM-ADD.NMR). The structure was subjected to a database search, and was found to be dihydroveratramine (DHV) (Fig. 1(III)).

4. Discussion

DHV was first synthesized by way of veratramine by Saito in 1940 [18], and was studied for its cardiac activity as an anti-accelerator in 1951 by Krayer et al. [19]. Apart from these papers, DHV has briefly been reported in the context of the more known veratramine [20–27]. Veratramine was reduced to DHV as earlier described with H_2 gas and a Pt catalyst [18]. The synthesized DHV shows MS^n spectra that precisely match the spectra of the here extracted compound. To our knowledge, DHV has not been observed as natural product earlier. We are confident that DHV was not a product of reduction of veratramine during the sample preparation, as a reduction of veratramineĭs double bond would be an energetically non-favored process under the conditions of the isolation procedure. In addition, DHV was detected by LC–MS in the crude benzene extract.

DHV possessed *in vitro* Hh inhibitory activity that is weaker than cyclopamine. DHV is similar to cyclopamine as it displays weaker inhibition when Hh signaling is activated by the SMO agonist SAG, when compared to Hh pathway activation by the morphogen Shh. Since the general Hh inhibitory activity of *V. album* ssp. *viriscens* extracts was rather high compared to the isolated DHV, it is likely that there are other active Hh pathway inhibitors present in the plant.

Since DHV, and possibly other ingredients in *V. album* ssp. *viriscens* affect Hh signaling, this study presents yet an argument that the use of *V. album* as an "alterative" medicine is highly questionable. Surprisingly, the plant is still found in some commercial products; one product containing *V. album* which has been marketed as an "infantile cholic remedy" has recently been associated with apparent life threatening events among infants [16].

5. Conclusions

In the *V. album ssp viriscens* subspecies, the modest Hh antagonist dihydroveratramine (DHV) was found in nature for the first time. From an analytical point of view, this study serves as an example of how one substance (DHV) can initially be mistaken for another (cyclopamine), even when employing LC–MS^n instrumentation.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.05.024.

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