Electronic Supplementary Material (ESI) for Chemical Communications

Exo-Cyclopamine – A Stable and Potent Inhibitor of Hedgehog-Signaling

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Supporting Information

Content:

1. General procedures.

A) Computational Details. The programs provided by the Turbomole-suite (ref. [1]) were applied for geometry optimization. The optimized structures were obtained employing the B97-D (ref. [2]) density functional and the resolution of identity approximation (ref. [3,4,5]). The def2-TZVP (ref. [6]) basis set was used throughout and the convergency criterion of the iteration cycle was increased to 10^{-8} Hartree. Solvation effects of ethanol were considered by applying the COSMO solvation model. (ref. [7])

The Gaussian03 (ref. [8]) program was used for the natural bond orbital (NBO) analysis (ref. [9]) with the TZVP (ref. [10]) basis set and the B3LYP (ref. [11,12,13]) functional. Solvation effects of ethanol were considered by applying the PCM (ref. [14]) solvation model.

B) Synthetic Chemistry. All reactions were run under an atmosphere of argon unless otherwise indicated. Room temperature refers to 22°C.

Reagents and anhydrous solvents were transferred via oven-dried syringe or cannula. Flasks were flame-dried under vacuum and cooled under a constant stream of argon. Dichloromethane was distilled under argon from SICAPENT (phosphorus pentoxide on solid support with indicator), and ethanol from magnesium. 1,2-Dimethoxyethane was purchased from Aldrich (anhydrous over molecular sieves).

All other chemicals were purchased from Sigma-Aldrich and Merck at highest commercially available purity and used as such.

Reactions were monitored by thin layer chromatography using Merck silica gel 60 F_{254} TLC aluminium sheets and visualised with ceric ammonium molybdate or vanillin staining solution. Chromatographic purification was performed as flash chromatography on Acros silica gel 35-70, 60 Å, using a forced flow of eluent (method of Still). Concentration under reduced pressure was performed by rotary evaporation at 40°C at the appropriate pressure.

Yields refer to chromatographically purified and spectroscopically pure compounds.

NMR spectra were recorded on a Varian Mercury plus 400 (operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C) spectrometer. Chemical shifts δ are reported in ppm with the solvent resonance as the internal standard (d_2-dichloromethane: 5.320 (^{1}H-NMR), 53.44 (^{13}C-NMR); $D_2O/CD_3OD/DCl$ (1:1, pH ~ 1.5): 4.656 (^{1}H-NMR). Coupling constants *J* are given in Hertz (Hz). Multiplicities are classified by the following abbreviations: s = singlet, d = doublet, or m = multiplet.

2D-spectra were recorded and allowed the complete assignment of all hydrogen- and carbon-atoms of a compound. The spectral data include this assignment using common steroid numbering. All spectra can be found as copies at the end of the experimental section.

High resolution mass spectra were obtained on a Bruker Daltonics ESI-FT-ICR-MS APEX II.

Melting points were measured on a Boetius-micro hot stage and are uncorrected.
Optical rotation data was obtained with a Schmidt+Haensch Polartronic MHZ-8 at the sodium-D line (589 nm) using a 50 mm path-length cell in the solvent and concentration indicated.

C) Biochemistry. The interference of cyclopamine and *exo*-cyclopamine, respectively, with the hh signaling pathway was tested in an established reporter gene assay (ref. [15]) based on the inhibition of the target gene *Gli1*. Shh-LIGHTII cells (ATCC CRL-2795, LGC, Wesel, Germany) represent a clonal mouse fibroblast cell line (NIH 3T3), which stably incorporates a *Gli*-dependent firefly luciferase reporter and a constitutive Renilla luciferase reporter. They were grown in 75 cm^2 cell culture flasks at 37°C in a humid atmosphere with 5 % CO_2. Cell growth medium DMEM (Dulbecco's Modified Eagle's Medium, high glucose, sodium pyruvate, w/o glutamine), Zeocin™ Selection Reagent and Geneticin® Selection Antibiotic (G418 sulfate) were obtained from Invitrogen. Additive L-glutamin and trypsin were obtained from PAA. Versene™ chelating agent was obtained from Gibco, bovine fetal serum (FBS) from Sigma. The cell freeze medium contained 5 % DMSO in complete growth medium.
The cell number was counted using a Neubauer-Zählkammer (Hemocytometer). DMEM (high glucose, sodium pyruvate, w/o glutamine) supplemented with 0.5 % FBS, 4 mM L-glutamin and 50 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH = 7.4) was used as incubation medium. The measurement of reporter gene and constitutive *Renilla* luminescence was performed with the Dual Luciferase® reporter system according to the manufacturer's instructions (Promega, Mannheim, Germany) using a GENios reader (TECAN, Crailsheim, Germany).

For achieving a qualitative characterization of cyclopamine and *exo*-cyclopamine, respectively, fertilized eggs from medaka were exposed to different compound concentrations. Medaka fishes were cultured at 26°C and a light:dark cycle of 14:10 hours. For breeding of medaka one female and one male each were placed in 3 L tanks in a circulation system. The eggs were collected within 1-2 hrs after fertilization by removing them from the female's belly.
For imaging of phenotypes caused by exposure, embryos were positioned in similar orientation and images were recorded with a digital consumer camera attached to a dissection microscope (Leica MZ16F, Wetzler, Germany).

2. Experiments.

A) Computational Studies.

We started our investigation by comparing the stability of possible conformations of *exo*-cyclopamine. The chair conformation (**3a** in Fig. 1) was the most stable structure, see table 1. However, both chair conformations, **3a** and **3b**, differ energetically only by 5 kJ/mol. Therefore, both conformations will be discussed in the following.

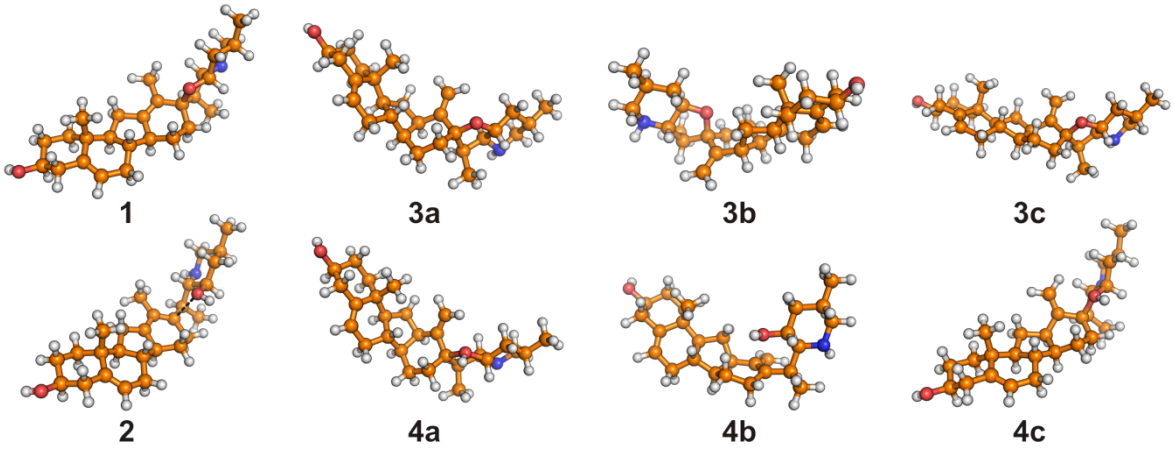


Figure 1: Ball-and-stick models of investigated structures. *top*: unprotonated cyclopamine derivatives; *bottom*: protonated cyclopamine derivatives.

A significant difference between cyclopamine and *exo*-cyclopamine can be found if the protonation of each compound is compared. While the proton transfer from hydronium cation to cyclopamine possesses a reaction energy of -96.4 kJ/mol (**1** + H_3O^+ → **2** + H_2O) it is only -44.9 kJ/mol for exo-cyclopamine (3a + H_3O^+ → 4b + H_2O). Please note, only trends and not absolute values should be compared to experiment because the calculation of charged particles in solution is still a challenge for computational chemistry. However, all investigated proton transfer reactions contain the same errors and therefore the large energy difference between the two reactions can also be expected for the experiment.

Table 1: Reaction energy ΔE in kJ/mol of the most stable *exo*-cyclopamine conformation **3a** to conformation **3x** (**3a** → **3x**) as well as the reaction energy ΔE of the protonation of **3a** forming **4a**, **4b** or **4c** (**3a** + H_3O^+ → **4x** + H_2O).

	3b	**3c**	**4a**	**4b**	**4c**
ΔE	5.3	9.7	–40.9	–44.9	–31.0

To gain insights upon the origin of the large energy difference between both proton transfer reactions we carried out a natural bond orbital (NBO) analysis (ref. [9]). The negative hyperconjugation obtained by the NBO analysis of σ-bonding orbitals into π-anti-bonding orbital of the protonated cyclopamine **2** is 93.8 kJ/mol while it is decreased to 13.4 kJ/mol for the protonated exo-cyclopamine **4b**, see Fig. 2 for illustration of hyperconjugation. This allows a stronger delocalisation of the π-bonding orbital into the unoccupied p-orbital at C17 for **2** (402.6 kJ/mol) than for **4b** (259.3 kJ/mol), see Fig. 3 for illustration.

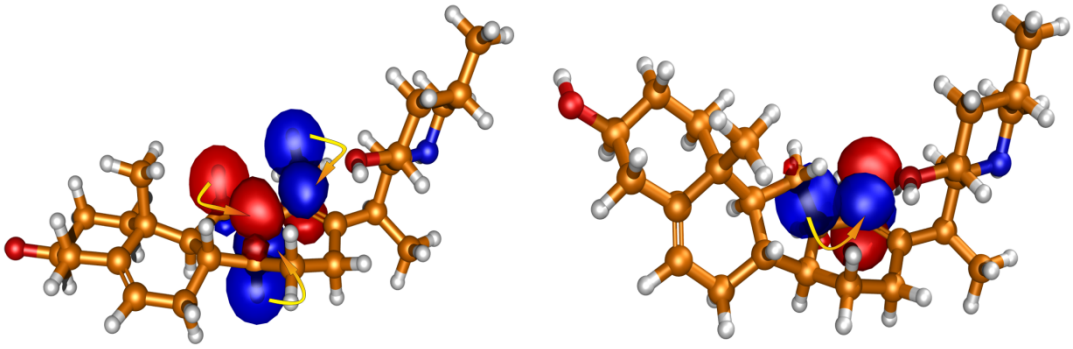


Figure 2: Natural bond orbitals showing the negative hyperconjugation of σ-bonding orbitals into π-anti-bonding orbital of cyclopamine (left) and *exo*-cyclopamine (right).

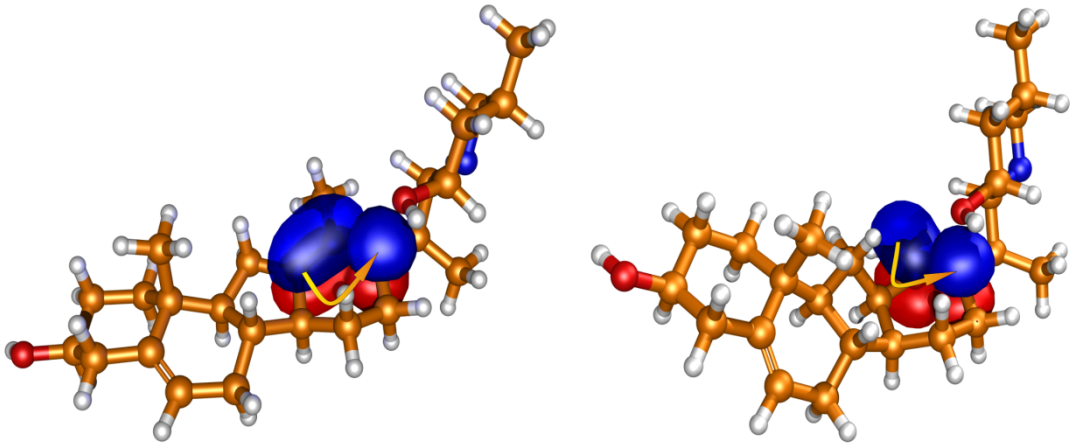


Figure 3: Natural bond orbitals showing the hyperconjugation of the π-bonding orbital with the unoccupied p-orbital at C17 for cyclopamine (left) and *exo*-cyclopamine (right).

Indications for the discussed hyperconjugation can also be observed from the Wiberg bond order (ref. [16]) and from the bond distance of selected atoms, see table 2. While for cyclopamine the lenght of the double bond $\mathbf{d}_{C12-C13}$ is increased by 5 pm and the bond order $\mathbf{BO}_{C12-C13}$ is 1.45 for the protonated derivative **2**, in *exo*-cyclopamine the double bond $\mathbf{d}_{C13-C18}$ is only increased by 2 pm and the bond order $\mathbf{BO}_{C13-C18}$ is 1.70 for the most stable protonated derivative **4b**. Furthermore, the bond distance $\mathbf{d}_{C11-C12}$ and $\mathbf{d}_{C12-C14}$ is sligthly

decreased from structure **1** to **2** while the Wiberg bond order $\mathbf{BO}_{C11-C12}$ and $\mathbf{BO}_{C12-C14}$ is increased. Furthermore, the stronger interplay of the occupied π-bonding orbital with the unoccupied p-orbital at C17 for **2** compared to **4b** can also be seen in the bond distance $\mathbf{d}_{C13-C17}$ and Wiberg bond order $\mathbf{BO}_{C13-C17}$. Finally, this results in a larger bond distance $\mathbf{d}_{C17-O1}$ and lower Wiberg bond order $\mathbf{BO}_{C17-O1}$ for **2** than for **4b**.

Table 2: Selected atom distances **d** in pm and Wiberg bond order **BO** of two atoms. Atom labels are shown in Fig. 4.

	1	**2**	**3a**	**3b**	**4a**	**4b**
$\mathbf{d}_{C11-C12}$	153	149	156	155	156	155
$\mathbf{BO}_{C11-C12}$	1.01	1.07	1.00	0.98	1.00	0.98
$\mathbf{d}_{C12-C13}$	134	139	153	152	153	152
$\mathbf{BO}_{C12-C13}$	1.82	1.45	1.00	1.01	0.99	1.00
$\mathbf{d}_{C12-C14}$	152	149	156	156	1.56	156
$\mathbf{BO}_{C12-C14}$	1.00	1.04	0.97	0.97	0.97	0.98
$\mathbf{d}_{C13-C17}$	153	141	154	154	152	145
$\mathbf{BO}_{C13-C17}$	0.99	1.36	0.97	0.97	1.00	1.21
$\mathbf{d}_{C13-C18}$	151	151	134	134	134	136
$\mathbf{BO}_{C13-C18}$	1.03	1.02	1.92	1.92	1.92	1.70
$\mathbf{d}_{C17-O1}$	149	268	146	148	162	244
$\mathbf{BO}_{C17-O1}$	0.87	0.05	0.90	0.87	0.68	0.13

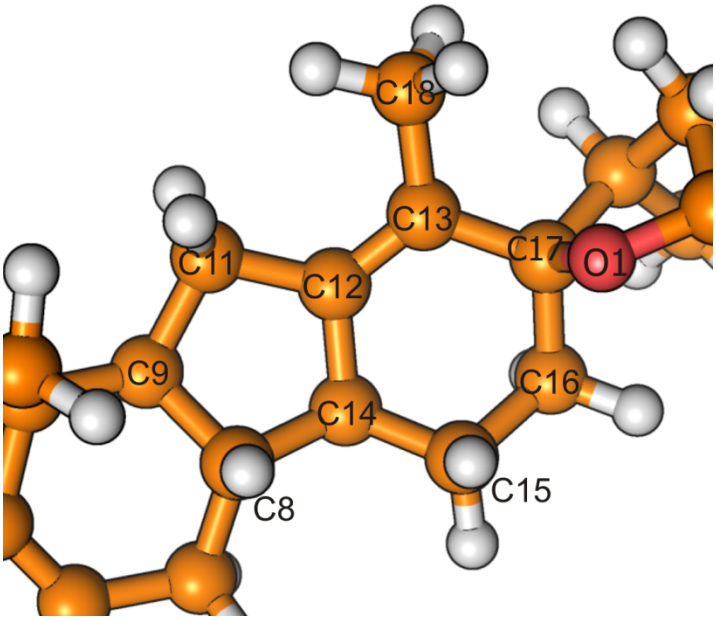


Figure 4: Labels of atoms.

The discussed hyperconjugation stabilizes **2** much more than **4b**, which might be the cause for the higher stability of *exo*-cyclopamine compared to cyclopamine. To prove this further, we investigated two model compounds **T1** and **T2**, see Fig. 5. **T2** possesses in contrast to **T1** all functional parts which participate in the hyperconjugation of cyclopamine and *exo*-cyclopamine. Therefore, the energy of the proton transfer reactions for **T2** should be comparable to the cyclopamine derivatives while for **T1** significantly reduced reaction energy should be obtained. As can be seen from table 3, for **1** + H_3O^+ → **2** + H_2O the reaction energy is about −95 kJ/mol for cyclopamine and **T2** while it is only −69.5 kJ/mol for **T1**. Contrary to this, the obtained reaction energies for the *exo*-cyclopamine models have nearly the same reaction energy for a proton transfer reaction (**3a** + H_3O^+ → **4x** + H_2O). These results confirm the role of the hyperconjugation illustrated in Fig. 2 and 3 for the higher stability of *exo*-cyclopamine compared to cyclopamine.

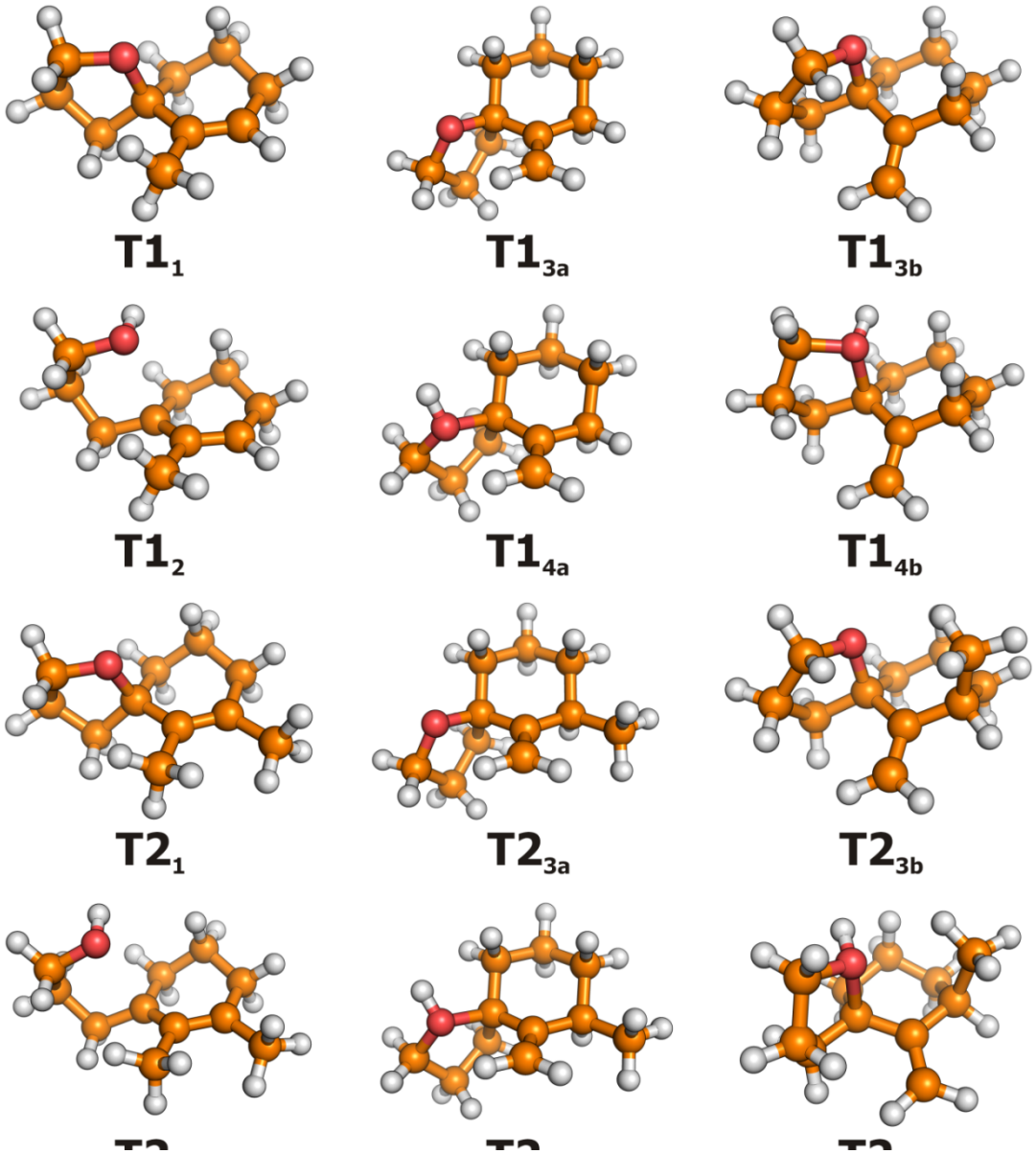
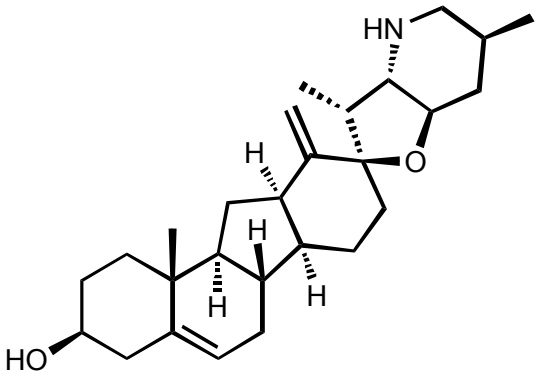


Figure 5: Investigated structures of model compounds **T1** and **T2**.

Table 3: Reaction energy ΔE in kJ/mol of conformation **3a** to conformation **3b** (**3a** → **3b**) as well as the reaction energy ΔE of the protonation of **1** forming **2** (**1** + H_3O^+ → **2** + H_2O) and the protonation of **3a** forming **4a** or **4b** (**3a** + H_3O^+ → **4x** + H_2O) for the cyclopamine derivatives and the two model compounds **T1** and **T2**.

	$\Delta E_{1 \longrightarrow 2}$	$\Delta E_{3a \longrightarrow 3b}$	$\Delta E_{3a \longrightarrow 4a}$	$\Delta E_{3a \longrightarrow 4b}$
cyclopamine	–96.4	5.3	–40.9	–44.9
T1	–69.5	5.9	–36.9	–37.1
T2	–92.2	11.8	–37.6	–30.7

B) Synthetic Chemistry, NMR-Experiments.



Exo-cyclopamine synthesis.

To a solution of *N*-Bs-*O*-Bn-*exo*-cyclopamine (8.0 mg, 12.5 μmol, ref. [17]) in EtOH (1 mL) freshly prepared Raney-Nickel (W2) (app. 0.25 g) in EtOH (2 mL) was added and the suspension was heated to reflux under vigorous stirring for 5 min. The mixture was allowed to cool to room temperature and filtered over a bed of Celite, washing several times with EtOH. The solvent was removed under reduced pressure, the crude material was redissolved in CH_2Cl_2 (2 mL) and filtered (paper), washing several times with CH_2Cl_2. The crude *N*-Bs-exo-cyclopamine was dried in vacuum for 12 hrs and then was redissolved in 1,2-dimethoxyethane (1 mL) and cooled to –78°C under stirring. To this a solution of freshly prepared sodium naphthalenide (0.5 M in DME, 250 μL, 125 μmol) was added dropwise and stirring at this temperature was continued for 30 min. Then, saturated aqueous $NaHCO_3$-solution (5 mL) was introduced, the suspension was allowed to warm to room temperature, and was extracted with CH_2Cl_2 (5 x 5 mL). The combined organic extracts were dried (Na_2SO_4), and all volatiles were removed under reduced pressure. Column chromatography (SiO_2; $CHCl_3$/EtOH/NH_3 (25 % aq.), 95:5:0.5) yielded pure title compound (3.6 mg, 8.2 μmol, 66 % over two steps) as a waxy solid.

m.p.: 215-225°C

R_f = 0.20 ($CHCl_3$/EtOH/NH_3(25 % aq.), 95:5:0.5)

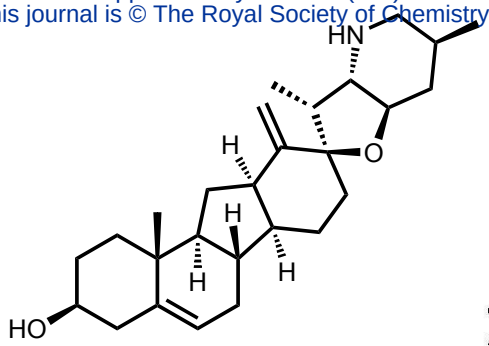
$[\alpha]_D^{22}$ = -44.2° (c = 0.17, CH_2Cl_2)

^{1}H-NMR (400 MHz, CD_2Cl_2) δ = 5.36 (m, 1 H, H-6), 5.33 (s, 1 H, H-18), 4.92 (s, 1 H, H-18), 3.50 (m, 1 H, H-23), 3.46 (m, 1 H, H-3), 3.03 (dd, *J* = 12.4, 4.2 Hz, 1 H, H-26), 2.71 (m, 1 H, H-12), 2.69 (m, 1 H, H-22), 2.34 (m, 1 H, H-4), 2.29 (m, 1 H, H-20), 2.27 (m, 1 H, H-26), 2.19 (m, 1 H, H-4), 2.18 (m, 1 H, H-24), 2.13 (m, 1 H, H-7), 1.86 (m, 1 H, H-16), 1.81 (m, 1 H, H-2), 1.79 (m, 1 H, H-11), 1.77 (m, 1 H, H-1), 1.74 (m, 1 H, H-11),

1.72 (m, 1 H, H-15), 1.65 (m, 1 H, H-25), 1.64 (m, 1 H, H-7), 1.62 (m, 1 H, H-16), 1.61 (m, 1 H, H-14), 1.53 (m, 1 H, H-2), 1.36 (m, 1 H, H-9), 1.35 (m, 1 H, H-8), 1.34 (m, 1 H, H-15), 1.24 (m, 1 H, H-24), 1.18 (m, 1 H, H-1), 1.03 (d, *J* = 7.4 Hz, 3 H, H-21), 1.01 (s, 3 H, H-19), 0.94 ppm (d, *J* = 6.7 Hz, 3 H, H-27).

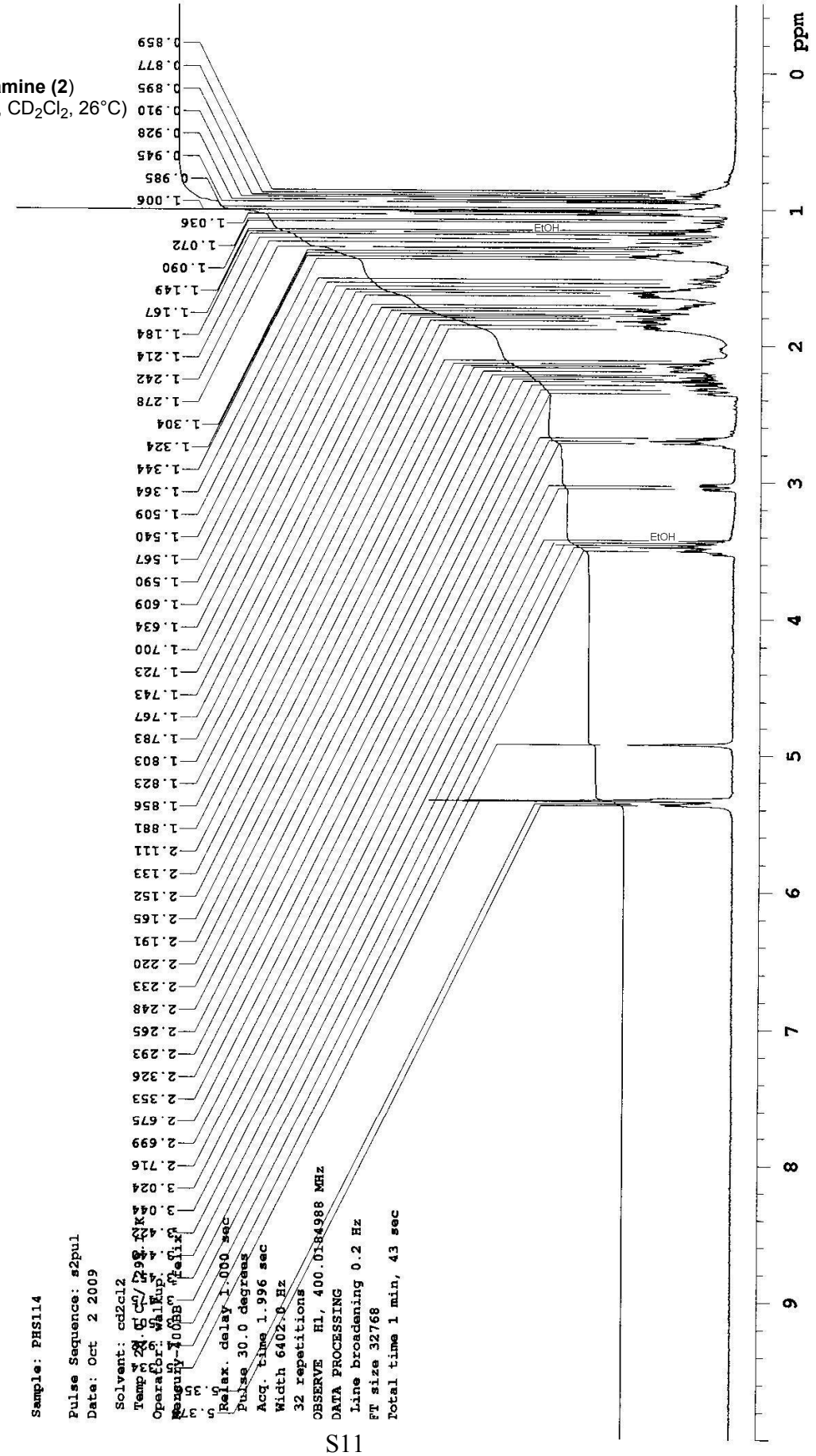
^{13}C-NMR (100 MHz, CD_2Cl_2) δ = 155.3 (C-13), 142.2 (C-5), 122.1 (C-6), 107.9 (C-18), 88.6 (C-17), 76.1 (C-23), 72.0 (C-3), 63.3 (C-22), 54.6 (C-26), 53.9 (C-9), 47.4 (C-14), 42.0 (C-4), 41.4 (C-12), 40.5 (C-20), 39.5 (C-24), 38.8 (C-8), 38.4 (C-1), 37.2 (C-10), 32.4 (C-16), 31.7 (C-2), 31.5 (C-25), 31.1 (C-7), 26.2 (C-11), 23.8 (C-15), 19.0 (C-19), 18.8 (C-27), 9.9 ppm (C-21).

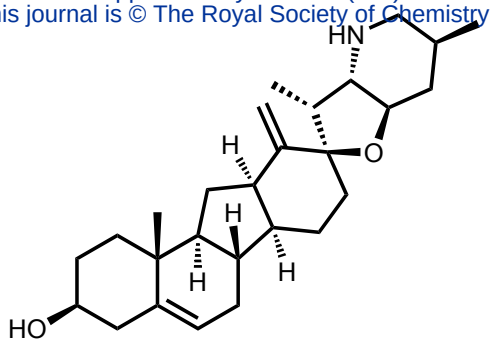
HRMS (*m/z*): $[M+H]^+$ calcd for $C_{27}H_{42}NO_2$: 412.32101, found: 412.32098.



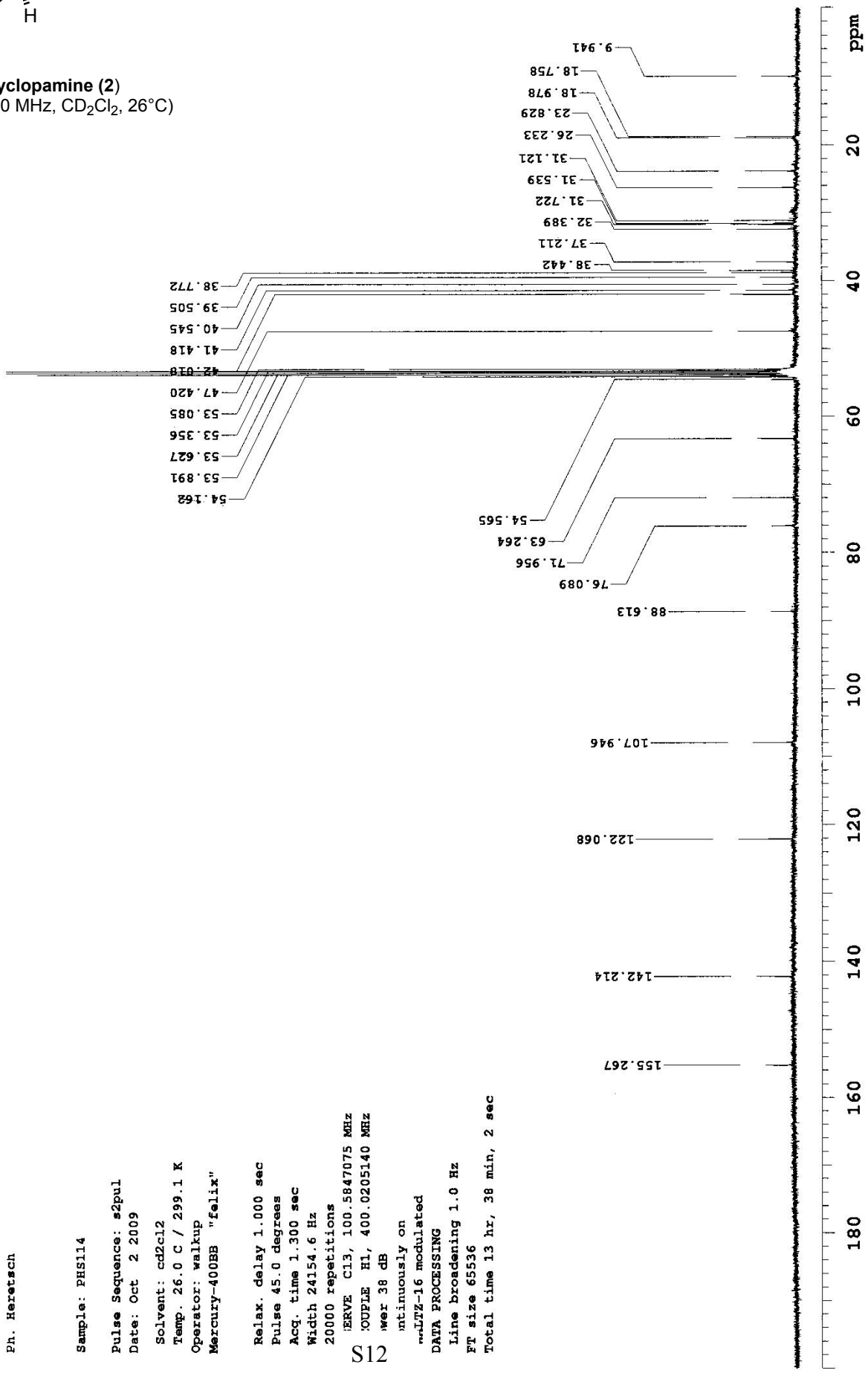
exo-cyclopamine (2)
^{1}H-NMR (400 MHz, CD_2Cl_2, 26°C)

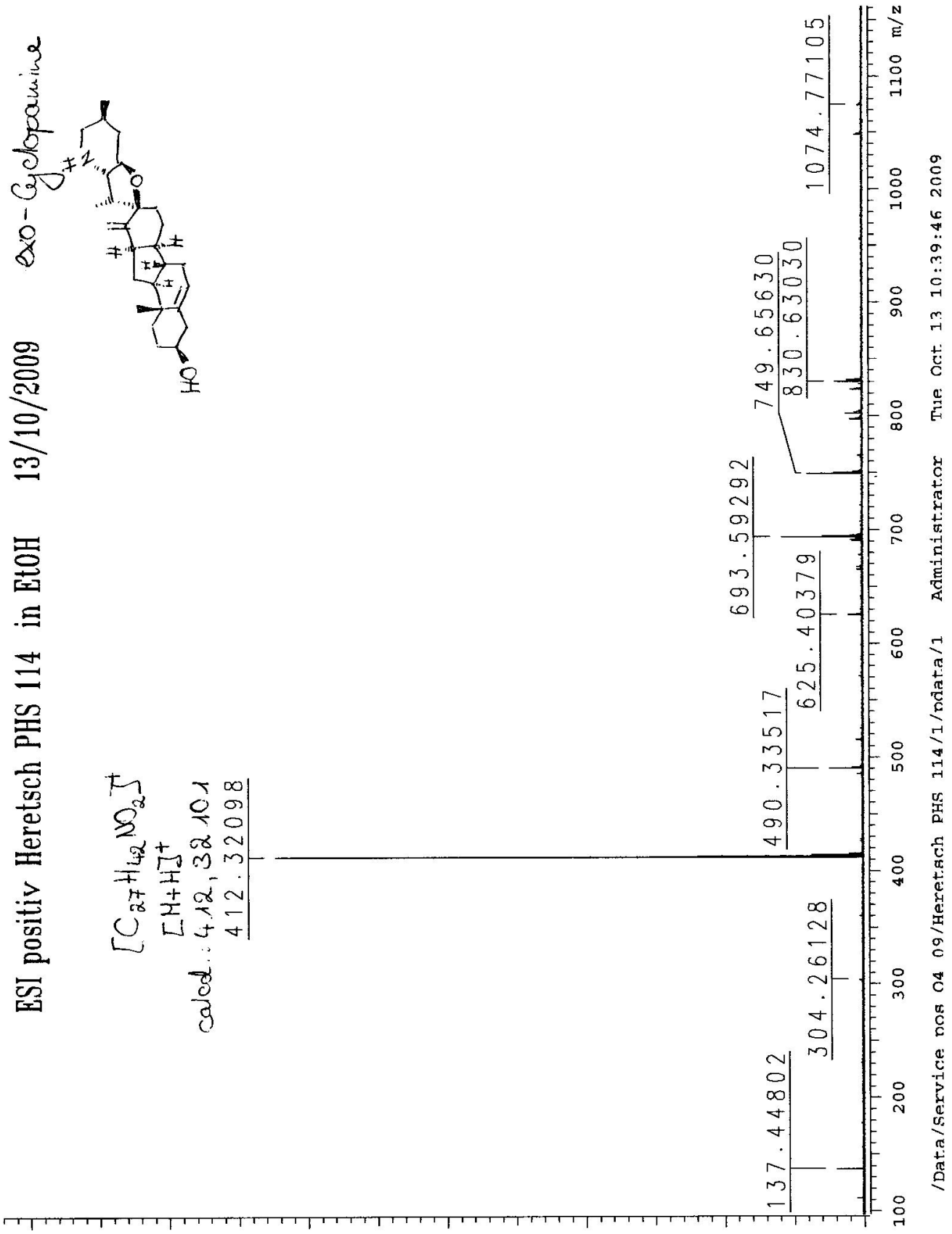
NMR-Experiments.





exo-cyclopamine (2)
^{13}C-NMR (100 MHz, CD_2Cl_2, 26°C)




ESI positiv Heretsch PHS 114 in EtOH 13/10/2009
exo-Cyclopamine
[C27H42NO2]+
[M+H]+
calcd.: 412,32201
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304.26128
490.33517
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749.65630
830.63030
1074.77105
a.i.
5.0e+07
4.0e+07
3.0e+07
2.0e+07
1.0e+07
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100
200
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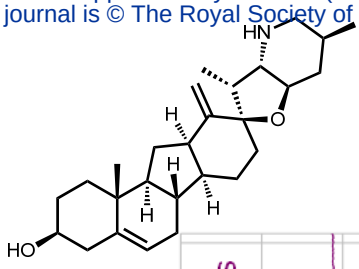
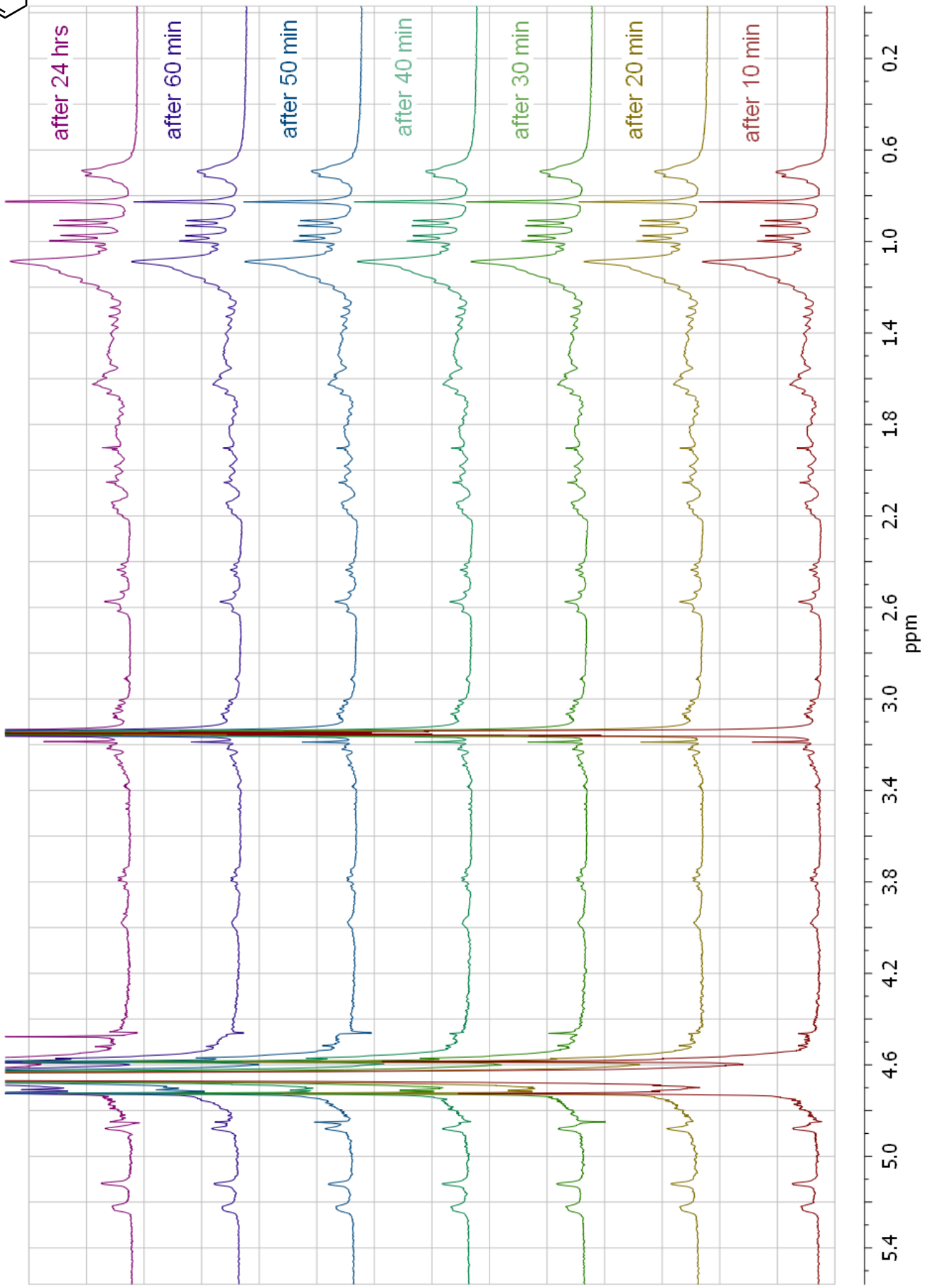
Studies on the stability of *exo*-cyclopamine and cyclopamine.

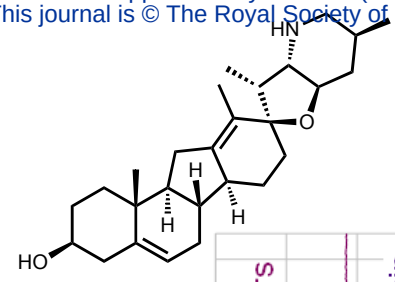
The sovent-system for the ^{1}H-NMR studies was prepared from D_2O (1.0 mL), CD_3OD (1.0 mL) and DCl (20 wt% in D_2O, 10 μL).

Exo-cyclopamine (1 mg) or cyclopamine (1 mg), respectively, were placed in a NMR tube each. A third NMR tube containing only the solvent system was initially used for locking and setting the residual water signal to 4.656 ppm. To the tube containing *exo*-cyclopamine the solvent system was then added (0.5 mL), taking this as a starting point (0 min), and the first spectrum was acquired after 10 min, then after 20 min, 30 min, 40 min, 50 min, and 60 min. The sample was then removed and the same was carried out for the tube containing cyclopamine, again taking spectra every 10 min over the course of 1 hr. Afterwards, both samples were stored in the dark, and after 24 hrs and locking with the solvent system the final spectra were acquired.

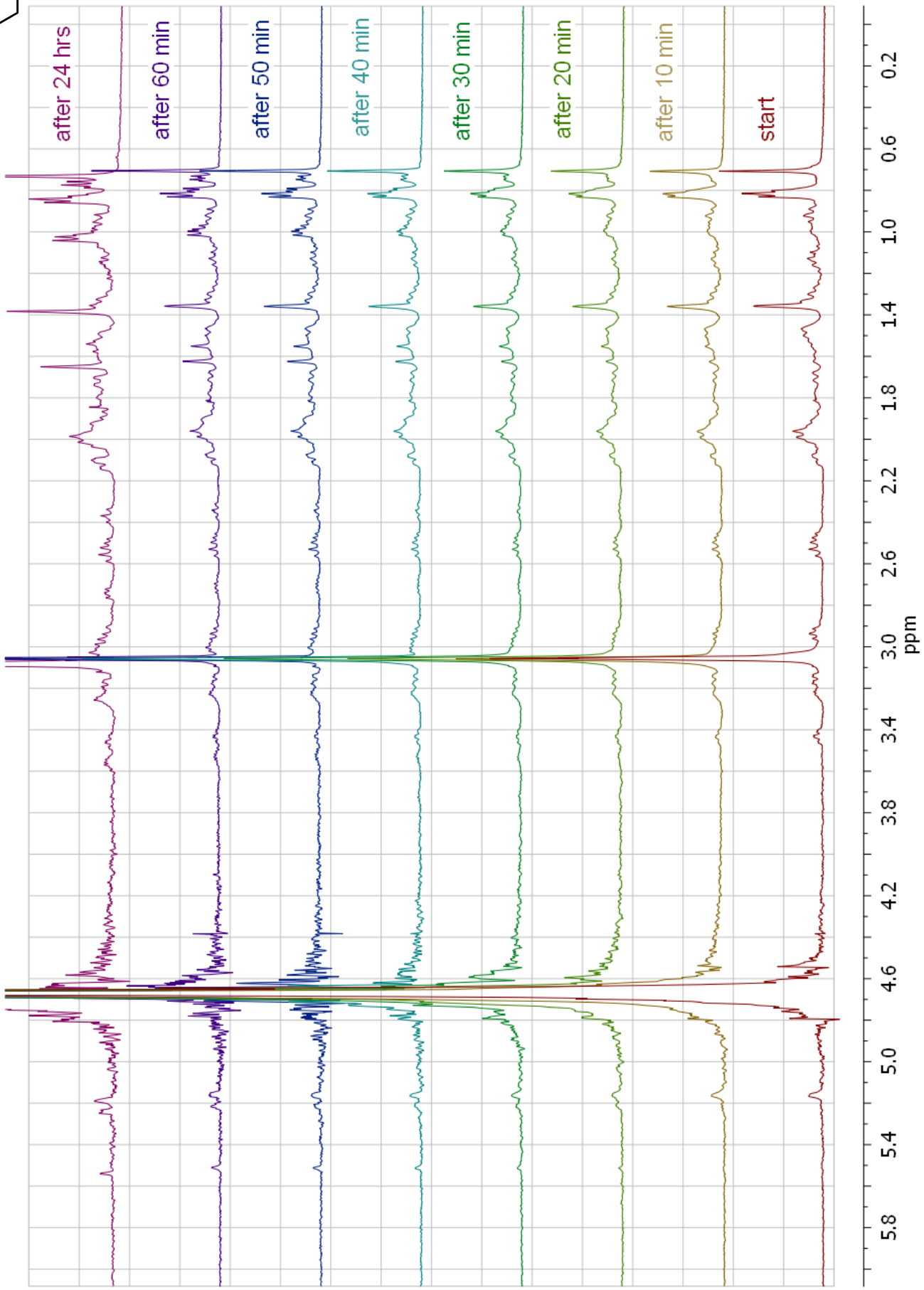


exo-cyclopamine (2)
^{1}H-NMR (300 MHz, $D_2O/CD_3OD/DCl$, 26°C)





cyclopamine (1)
^{1}H-NMR (300 MHz, $D_2O/CD_3OD/DCl$, 26°C)



C) Biochemistry.

Luciferase reporter assay.

Incubation of cells.

For performing the assay, Shh-LIGHTII cells were grown to reach 80 % confluence, washed twice with Versene, detached with 2 mL trypsin for not longer than 2 min and resuspended in 8 mL of growth medium. After centrifugation for 3 min, the supernatant was removed and the cell pellet dissolved in approximately 10 mL of growth medium. Finally, 20,000 to 100,000 cells per well were cultured in a 24-well plate for 48 hrs.

For exposure to cyclopamine and *exo*-cyclopamine the growth medium was removed and the cells were exposed to the compound in 500 µL/well incubation medium for 48 hrs. Stock solutions were prepared in EtOH and introduced in different concentrations into the incubation medium to reach a final solvent concentration of 0.05 %. Due to the assay principle, Shh-LIGHTII cells had to be co-exposed to 100 nM of SAG (Smoothened agonist) for determining the inhibitory activity of the compounds. A SAG stock solution was prepared in EtOH and introduced into the medium. The final EtOH-concentration – introduced by SAG and the test compound – was 0.1 %. As a positive control a 100 nM SAG solution in incubation medium (0.1 % EtOH) was used, negative controls were treated with EtOH only.

Performing the luciferase measurement.

After 48 hrs of incubation cells were washed with 500 µL PBS (phosphate buffered saline, w Mg/Ca, Invitrogen) and afterwards incubated for 15 min with 100 µL 1x passive lysis buffer on a shaker. The lysed cells together with lysis solution were transferred into 1.5 mL reaction tubes, centrifuged for 1 min at 4°C and kept on ice until further use. At first, the blank of luminescence of a 96-well plate (flat bottom, white, Greiner bio-one) was recorded. Then, 100 µL luciferase assay reagent and 10 µL cell supernatant were mixed per well and the luminescence of firefly luciferase was recorded. Addition of 100 µL Stop&Glo® reagent permitted the recording of Renilla luminescence. The measurement was performed per row of a well, i.e. assay reagent was added to 8 wells and luminesescence recorded, before the next row was proceeded.

Analysis of constitutive Renilla luminescence was used to normalize for any potential unspecific *Gli1*-reporter gene luminescence.

Evaluation of IC_{50}.

For determination of IC_{50} values, the relative luminescence units per second RLU/s (relative luminescence units per second, quotient of firefly and *Renilla* luminescence) were plotted

against the inhibitor concentrations on a log10 scale. Each test was performed three times with three replicates per concentration.

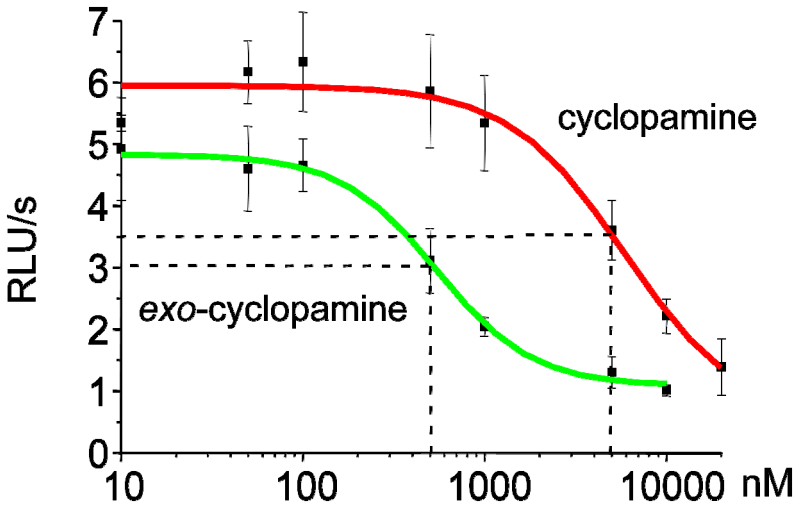


Figure 6: Comparison of IC_{50}-values of Shh inhibition by *exo*-cyclopamine (IC_{50} = 0.5 µM) and cyclopamine (IC_{50} = 5 µM) in a *Gli1*-reporter gene assay. Data were obtained from three independent experiments. Data represent the mean ± standard deviations.

Exposure of fish embryos for cyclopia phenotype test.

Treatment with inhibitors.

The exposure media were prepared as follows: a compound stock solution was prepared in 100 % EtOH. Subsequently, these solutions were added to 5 mL local tap water (pH = 8-8.2, water hardness 1.2-2.4 mM bivalent ions, conductivity 520-560 mS/cm) supplemented with 0.1 % methylene blue (Merck) to reach final EtOH concentration of 0.1 %. Exposure started at 4-5 hrs post fertilization with 30-40 medaka eggs per incubation flask (10 mL screw capped glas vessels) and was continued until 5 d post fertilization at 26°C. Control embryos were treated with 0.1 % EtOH only in local tap water (containing 0.1 % methylene blue).

Microscopical analysis and cyclopia assay.

The average eye distance in treated medaka embryos was measured for at least 30 eggs per concentration by comparison to a reference scale. The stage of development was identified according to ref. [18].

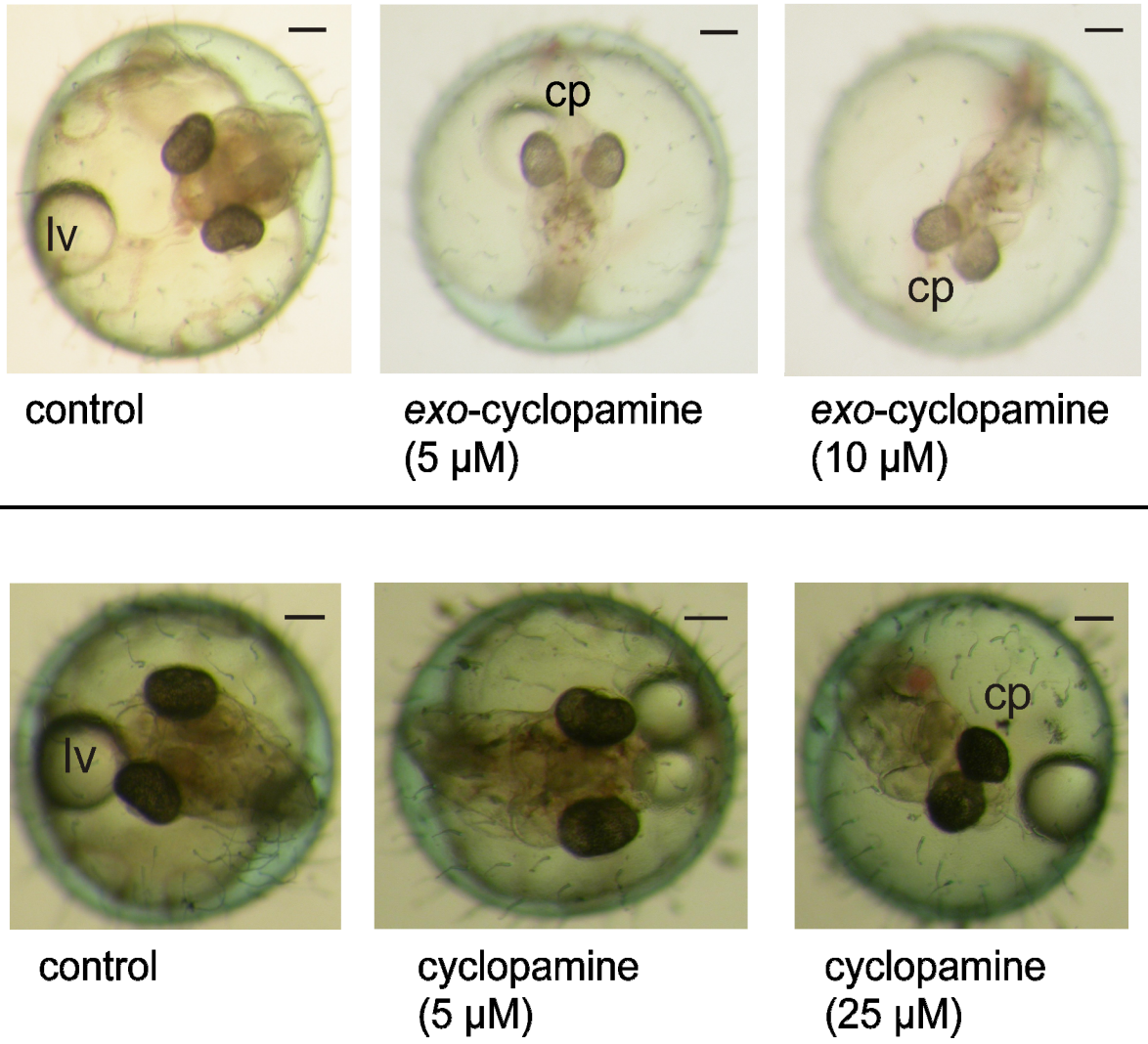


Figure 7: Development of medaka embryos exposed to either *exo*-cyclopamine or cyclopamine. Exposure induced the cyclopia phenotype (indicated by a reduced distance of the eyes, cp). All images were recorded at 5 days post fertilization and are representative examples of 30 embryos exposed per concentrations. The scale bar represents 100 µm. cp, cyclopia; lv, lipid vesicle.

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