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Cyclopamine analogs bearing exocyclic methylenes are highly potent and acid-stable inhibitors of hedgehog signaling

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Full Research Paper

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Abstract

The chemical synthesis and biological evaluation of new cyclopamine analogs bearing exocyclic methylenes in different positions is described. Bis-*exo*-cyclopamine **6** was identified as a potent inhibitor of the Gli1-dependent luciferase expression in Shh-LIGHTII cells. An extension of this study to F-ring-modified structures shows the necessity of a rigidly positioned nitrogen atom for bioactivity as well as the presence of the C21 methyl group for acid stability and bioactivity.

Introduction

Hedgehog signaling is involved in embryonic development and plays an important role in the maintenance of stem cells, tissue repair and regeneration in adult organisms [1-4]. The erroneous activation of hedgehog signaling is tightly associated with the occurrence of basal cell carcinoma and medulloblastoma [5]. In addition, several other tumors are co-dependent on hedgehog signaling, examples for this type are cancers of the skin [6,7], brain [8,9], lung [10], pancreas [11], breast [12], prostate [13,14], colon [15], rhabdomyosarcoma [16], lymphoma [17-19], multiple myeloma [17,20], and chronic myeloic leukemia [21-23]. More recently, other diseases like diabetes [24,25], neurodegenerative disorders [26], and trisomy [27,28], have been linked with hedgehog signaling.

Cyclopamine (**1**, see Figure 1) was the first inhibitor of the hedgehog signaling pathway to be identified. As a highly selec-

tive inhibitor of the transmembrane protein Smoothened (Smo), an integral component of hedgehog signaling, it presents an attractive target for medicinal and pharmaceutical research [29,30]. Unfortunately, its direct development into a drug is hampered by its low metabolic stability (decomposition at pH < 3) [31] and rather moderate potency (IC_{50} ~ 5 μM).

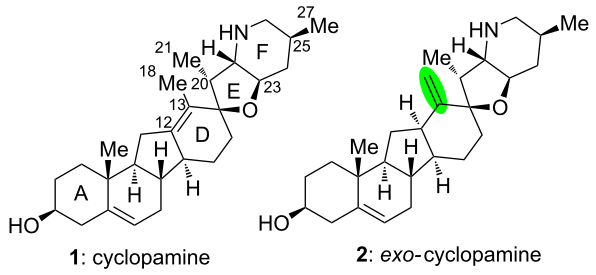


Figure 1: Structures of cyclopamine and *exo*-cyclopamine.

We previously reported the first chemical synthesis of cyclopamine (**1**) starting from dehydroepiandrosterone and utilizing the C–H-functionalization logic and a biomimetic skeleton rearrangement [32,33]. Furthermore, quantum mechanical calculations guided our design and synthesis of *exo*-cyclopamine (**2**, see Figure 1), a ten-fold more potent and acid-stable analog with an *exo*-methylene unit at C13–C18 [34]. Herein, we describe a comprehensive study of cyclopamine analogs bearing *exo*-methylene units in different positions and extent this rational to F-ring-modified structures.

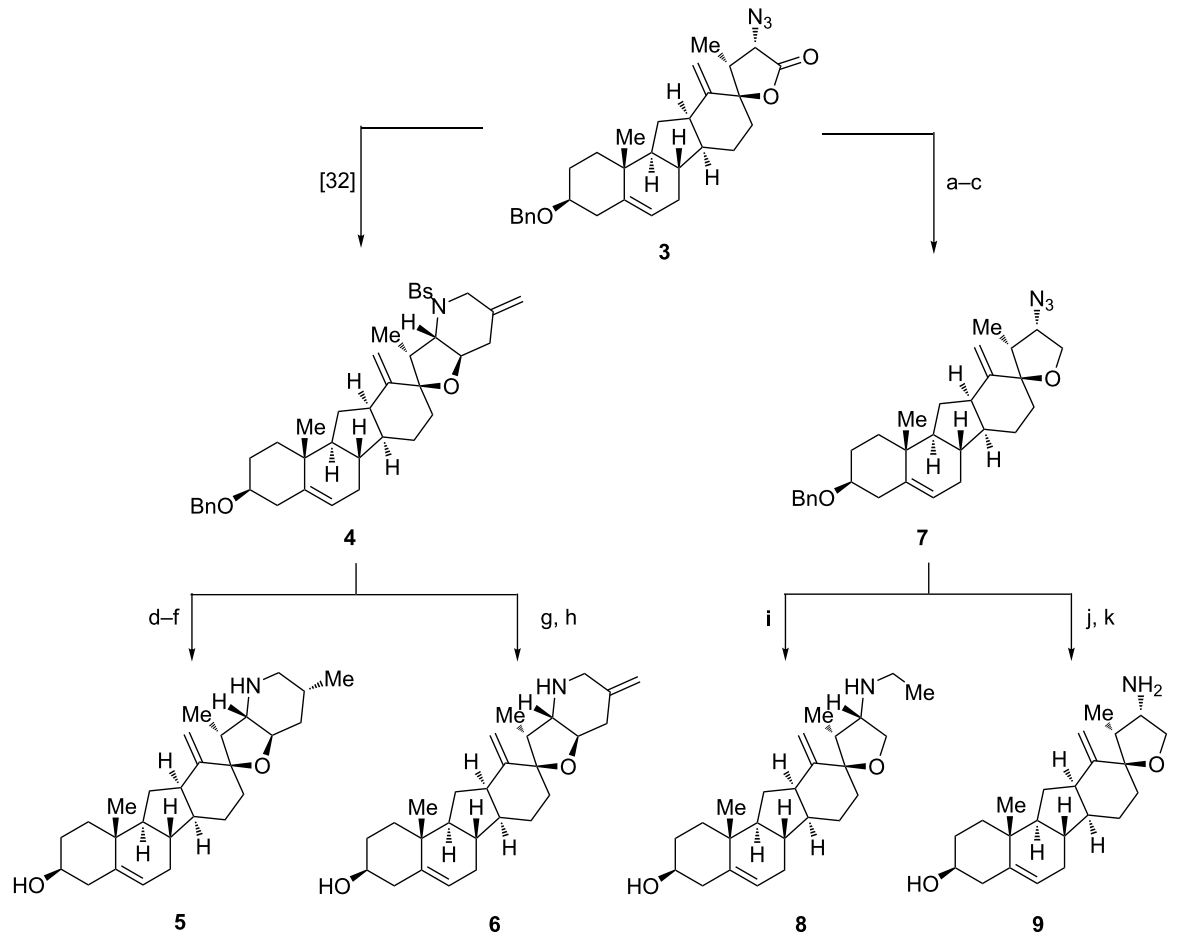
Results and Discussion

Our synthetic approach to the analogs described here started from previously reported azide **3** which was converted in six steps [32] to the protected bis-*exo*-methylene compound **4**. Carefully chosen conditions for the deprotection of the benzyl ether (DDQ, DCE/pH 7 phosphate buffer, 40 °C, 86%) and the benzenesulfonylamine (sodium naphthalenide, DME, −78 °C, 79%) allowed for the isolation of bis-*exo*-cyclopamine **6** in 18% overall yield from **3**. The hydrogenation of intermediate **4** by using Wilkinson's catalyst in benzene yielded previously described *exo*-cyclopamine **2** and its C25 epimer **5**. Deprotection with Raney-nickel (EtOH, 78 °C) and then sodium naphthalenide (DME, −78 °C, 41% over two steps) furnished 25-epi-*exo*-cyclopamine **5** in 3% overall yield from **3**.

A first set of *exo*-cyclopamine analogs with a modified F-ring was obtained starting again from azide **3** (see Scheme 1). Reduction of the lactone moiety to give a tetrahydrofuran was accomplished in three steps including (1) partial reduction to the lactol (DIBAl-H, THF, −78 °C to −60 °C, 95%), (2) acetylation (Ac_2O, pyridine, cat. DMAP, quant.) and (3) reductive removal of the so-obtained acetate (Et_3SiH, $BF_3{\cdot}Et_2O$, −78 °C to −20 °C, 79%). Removal of the benzyl ether, reduction of the azide moiety and concomitant alkylation was then all effected in one pot by using Raney-nickel in EtOH to give analog **8**, an F-ring-opened *exo*-cyclopamine derivative in 27% overall yield from **3**. Alternatively, the use of previously devised conditions for benzyl deprotection (DDQ, DCE/pH 7 phosphate buffer, 45 °C, 78%) and then sodium borohydride-mediated reduction of the azide (EtOH, 65 °C, 62%) cleanly furnished primary amine **9**, an *exo*-cyclopamine derivative with no F-ring (35% overall yield from **3**).

A second set of F-ring-modified *exo*-cyclopamine derivatives was obtained starting from previously described 12-ß-triethylsilyloxy compound **10** (see Scheme 2) [32]. To construct the spirolactone **12**, a three-step process consisting of (1) allylation (allylcerium chloride, THF, 0 °C, 93%), (2) hydroboration/oxidation (9-BBN, THF, 70 °C; then $NaBO_3$, H_2O, 50 °C, 91%) and finally, (3) oxidative cyclization (BAIB, cat. TEMPO, CH_2Cl_2, 25 °C, 73%) was employed. Deprotection of the silyl ether (HF, MeCN, 25 °C, 87%) and base-induced rearrangement of the steroid skeleton via the corresponding triflate (Tf_2O, pyridine, 0 °C→50 °C, 46%) gave C-*nor*-D-*homo*-derivative **13** which, in turn, was transformed into lactone azide **14** by using Evans' conditions (LDA, THF, −78 °C to −30 °C, 10 min; then trisylazide, THF, −78 °C, 1.5 h, 43%) [35]. From **14** two additional *exo*-cyclopamine derivatives became accessible. Partial reduction to the lactol (DIBAl-H, THF, −78 °C to −65 °C, 88%) and Horner–Wadsworth–Emmons reaction by using previously reported dimethyl {3-[(4-methoxybenzyl)oxy]-2-oxopropyl}phosphonate [32], ($Ba(OH)_2$, THF/H_2O, 80 °C, 50%) led to **15** which, in turn, was methylenated by employing the Peterson protocol ($TMSCH_2CeCl_2$, THF, −78 °C; then TMEDA; then HF, MeCN, 25 °C, 64%) [36]. Towards this end the azide moiety was reduced and monoprotection of the so-obtained amine as a sulfonamide ($LiAlH_4$, THF, 0 °C→25 °C; then benzenesulfonyl chloride, Et_3N, DMF, 0 °C, 69%) gave **17**. Deprotetion of the 4-methoxybenzyl ether (DDQ, DCE/pH 7 phosphate buffer, 25 °C, 59%) and cyclization under Mitsunobu conditions (*n*-Bu_3P, DEAD, toluene, 0 °C→25 °C, 93%) yielded piperidine **18**. Deprotection of the benzyl ether by using previously devised conditions (DDQ, DCE/pH 7 phosphate buffer, 44 °C, 70%) and the benzenesulfonylamine (sodium naphthalenide, DME, −78 °C, 95%) gave 20-demethyl-bis-*exo*-cyclopamine **19** in 7% overall yield starting from **14**.

Starting from azido lactone **14** (see Scheme 2) a partial reduction to the lactol (DIBAl-H, THF, −78 °C to −65 °C, 88%) and the Horner–Wadsworth–Emmons reaction by using triethyl phosphonoacetate ($Ba(OH)_2$, THF/H_2O, 80 °C, 55%) led to

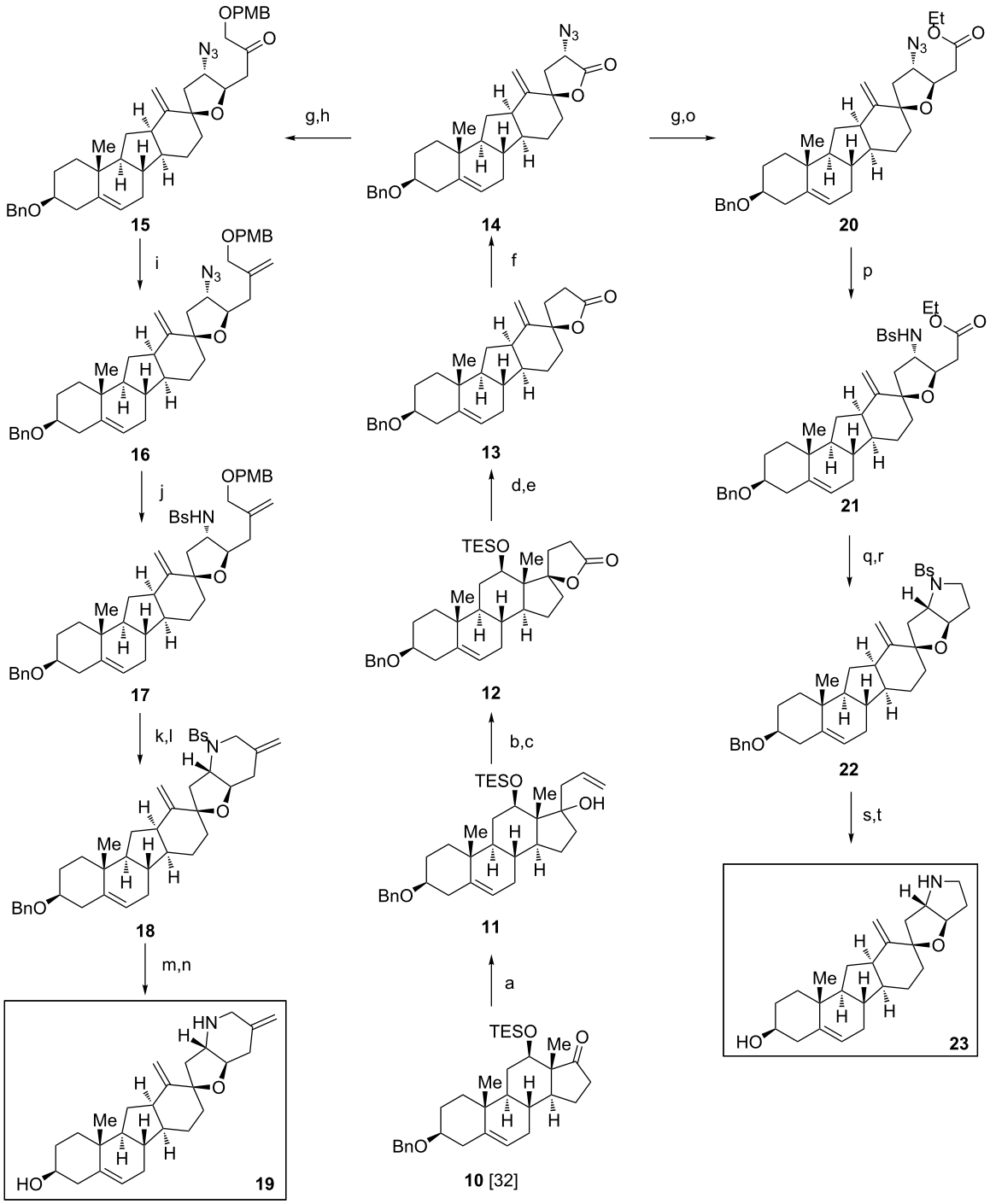


Scheme 1: Synthesis of 25-epi-*exo*-cyclopamine **5**, bis-*exo*-cyclopamine **6**, and derivatives **8** and **9**. Reaction conditions: (a) DIBAl-H, THF, −78 °C to −65 °C, 4 h, 95%; (b) Ac_2O, pyridine, DMAP (cat.), 25 °C, 20 h, quant; (c) $BF_3{\cdot}Et_2O$, Et_3SiH, CH_2Cl_2, −78 °C to −20 °C, 7 h, 79%; (d) $Rh(PPh_3)_3Cl$, benzene, H_2, 25 °C, 24 h, quant. (3:1 dr); (e) Raney-nickel (W2), EtOH, 78 °C, 5 min; (f) sodium naphthalenide, DME, −78 °C, 30 min, 41% over two steps; (g) DDQ, DCE/phosphate buffer (pH 7), 40 °C, 95 min, 86%; (h) sodium naphthalenide, DME, −78 °C, 1 h, 79%; (i) Raney-nickel (W2), EtOH, 37%; (j) DDQ, DCE/phosphate buffer (pH 7), 45 °C, 1.5 h, 78%, (k) $NaBH_4$, EtOH, 65 °C, 5 d, 62%.

ethyl ester **20**. Reduction of the azide moiety in **20** was carried out by using Staudinger's protocol (Ph_3P, THF/H_2O, 50 °C). Immediate protection of the obtained amine (benzenesulfonyl chloride, Et_3N, CH_2Cl_2, 40 °C, 97% over 2 steps) gave sulfonylamide **21**. Reduction of the ester in **21** to the primary alcohol (DIBAl-H, THF, −78 °C to −40 °C, 97%) and cyclization by employing Mitsunobu conditions (*n*-Bu_3P, DEAD, toluene, 0 °C→25 °C, 81%) yielded pyrrolidine **22**. Previously devised conditions for the deprotection (1. DDQ, DCE/pH 7 phosphate buffer, 40 °C; 2. sodium naphthalenide, DME, −78 °C, 46%) finally afforded F-*nor*-20,25-bis-demethyl-*exo*-cyclopamine **23** in 9% overall yield starting from **14**.

Next, we examined all synthesized compounds for their ability to inhibit the Gli1-dependent luciferase expression in Shh-LIGHTII cells, a clonal mouse fibroblast cell line which stably incorporates a Gli-dependent firefly luciferase reporter and a constitutive *Renilla* luciferase reporter [37]. The compounds were tested in a concentration range from 0.01 μM to 10 μM. While analogs **8**, **9**, and **19** showed no activity (data not shown) in this concentration range, **5**, **6**, and **23** were active with 25-epi-*exo*-cyclopamine **5** having an IC_{50} of 3.29 ± 0.31 μM, F-*nor*-20,25-bis-demethyl-*exo*-cyclopamine **23** of 6.4 ± 0.9 μM, and bis-*exo*-cyclopamine **6** being the most active with an IC_{50} of 0.20 ± 0.01 μM (see Figure 2).

Given the negative test results of compounds **8** and **9** it becomes evident that the F-ring is necessary for bioactivity. The piperidine moiety provides a rather rigidly placed nitrogen atom. Nevertheless, a pyrrolidine as in compound **23** still provides the correct orientation of the nitrogen atom. Despite compounds **8** and **9** being inactive in the assay, both derivatives induced cytotoxicity in the concentration range tested. Very subtle changes of the conformation of the piperidine ring significantly change



Scheme 2: Synthesis of 20-demethyl-bis-*exo*-cyclopamine **19** and F-*nor*-20,25-bis-demethyl-*exo*-cyclopamine **23**. Reaction conditions: (a) allylcerium chloride, THF, 0 °C, 30 min, 93%; (b) 9-BBN, THF, 70 °C, 6 h; then $NaBO_3$, 50 °C, 12 h, 91%; (c) BAIB, TEMPO, CH_2Cl_2, 25 °C, 3 h, 73%; (d) HF 50 wt % in H_2O, MeCN, 20 min, 25 °C, 87%; (e) Tf_2O, pyridine, 0 °C→50 °C, 1.5 h; then additional Tf_2O, 0 °C→50 °C, 2 h, 46%; (f) LDA, THF, −78 °C to −30 °C, 10 min; then trisylazide, THF, −78 °C, 1.5 h, 43%; g) DIBAl-H, THF, −78 °C to −65 °C, 2 h, 88%; (h) dimethyl (3-((4-methoxybenzyl)oxy)-2-oxopropyl)phosphonate, $Ba(OH)_2$, THF/H_2O, 80 °C, 13 h, 50%; (i) (trimethylsilyl)methylcerium chloride, THF, −78 °C, 30 min; then TMEDA, −78 °C, 15 min; then HF 50 wt % in H_2O, MeCN, 25 °C, 10 min, 64%; (j) $LiAlH_4$, THF, 0 °C→25 °C, 13 h; then benzenesulfonyl chloride, Et_3N, DMF, 0 °C, 25 min, 69%; (k) DDQ, CH_2Cl_2/phosphate buffer (pH 7), 25 °C, 2 h, 59%; (l) *n*-Bu_3P, DEAD, toluene, 0 °C→25 °C, 12 h, 93%; (m) DDQ, DCE/phosphate buffer (pH 7), 44 °C, 50 min, 70%; (n) sodium naphthalenide, DME, −78 °C, 30 min, 95%; (o) triethyl phosphonoacetate, $Ba(OH)_2$, THF, 80 °C, 12 h, 55%; (p) PPh_3, THF/H_2O, 50 °C, 24 h; then benzenesulfonyl chloride, Et_3N, CH_2Cl_2, 40 °C, 5 h, 97%; (q) DIBAl-H, THF, −78 °C to −40 °C, 3 h, 97%; (r) *n*-Bu_3P, DEAD, toluene, 0 °C→25 °C, 24 h, 81%; (s) DDQ, DCE/phosphate buffer (pH 7), 40 °C, 30 min, 62%; (t) sodium naphthalenide, DME, −78 °C, 40 min, 74%.

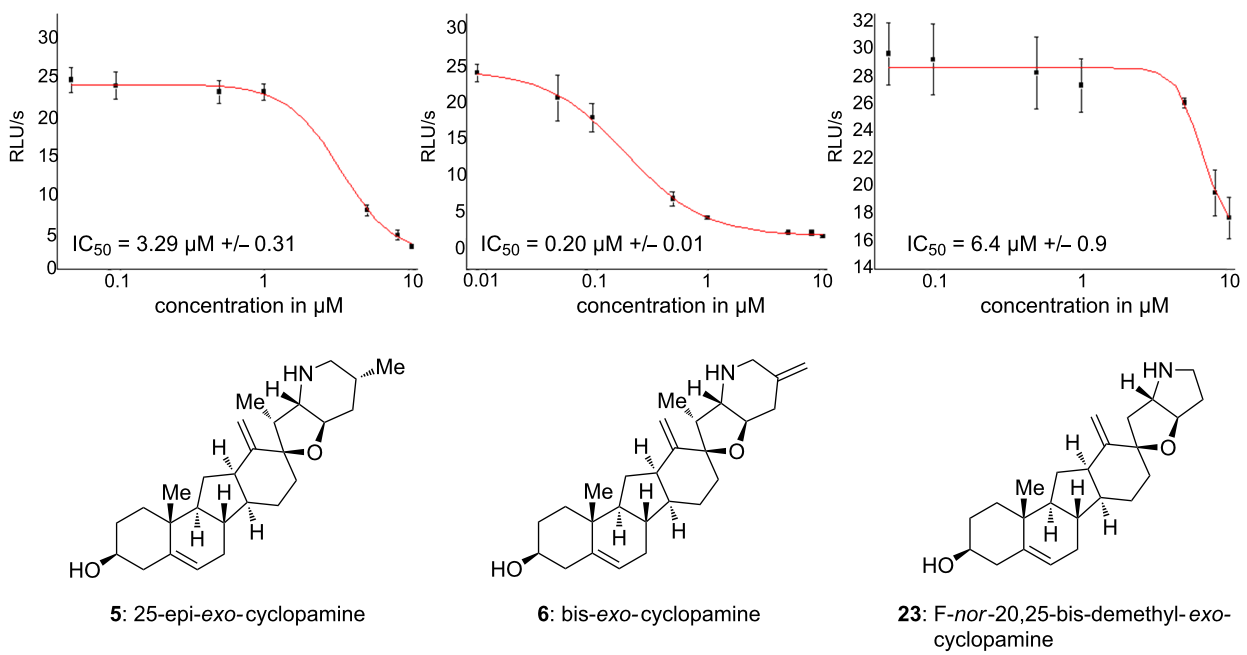


Figure 2: IC_{50} values of Shh inhibition by compounds **5**, **6** and **23** in a Gli1-reporter gene assay. Data were obtained from three independent experiments and represent the mean ± standard deviation.

bioactivity: While 25-epi-*exo*-cyclopamine **5** shows reduced activity in comparison to *exo*-cyclopamine **2**, bis-*exo*-cyclopamine **6** is the most active compound tested in this study. Furthermore, the methyl group at C-20 seems to have a pronounced effect on the bioactivity, with 20-demethyl-bis-*exo*-cyclopamine **19** being completely inactive in the tested concentration range.

Finally, we studied the stability of all newly synthesized compounds towards acidic conditions. Therefore, they were exposed to a pH of approximately 1 (MeOH, 1 M HCl) for 24 h. After evaporation of all volatiles, ^{1}H NMR spectra were acquired and compared to the initially obtained spectra of the pure compounds. While compounds **5**, **6**, **8**, and **9** remained unchanged, compounds **19** and **23** showed decomposition. This experiment emphasizes the importance of the C-21 methyl group for the stability of *exo*-cyclopamine derivatives. All synthesized compounds, the number of steps required, and the respective overall yield starting from **3** or **14**, as well as their biological activity and stability under acidic conditions are summarized in Table 1.

Conclusion

In conclusion, we succeeded in identifying the new cyclopamine derivative bis-*exo*-cyclopamine **6** which surpasses the biological potency of the parent compound by the 25-fold and is stable at pH 1. Further insights were gained into the structure–activity relationship of F-ring-modified analogs of cyclopamine and the necessity of the C-21 methyl group for

Table 1: Synthesized *exo*-cyclopamine derivatives, number of steps, yields, results of their biological testing, and stability towards acid.

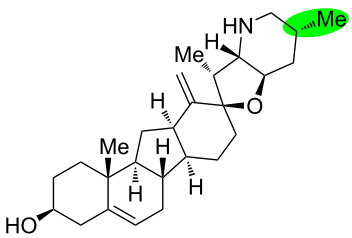
Compound	Number of steps from **3** or **14**	Overall yield	Potency in Gli-assay	Stability towards acid (pH ~1, 24 h)
 **5**: 25-epi-*exo*-cyclopamine	9 steps from **3**	3%	3.29 ± 0.31 μM	stable

Table 1: Synthesized *exo*-cyclopamine derivatives, number of steps, yields, results of their biological testing, and stability towards acid. (continued)

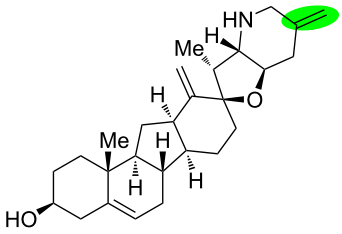
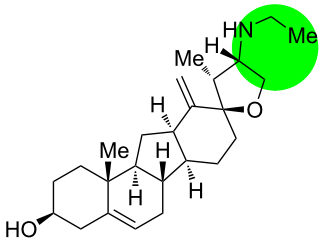
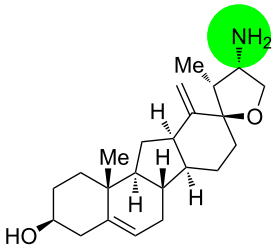
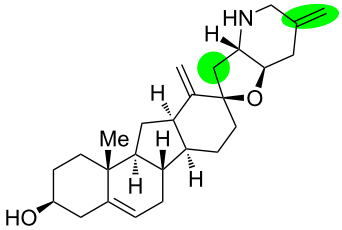
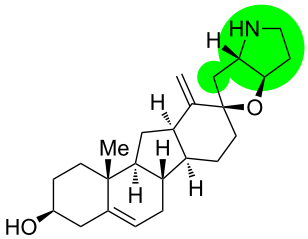
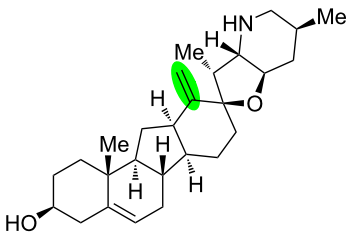
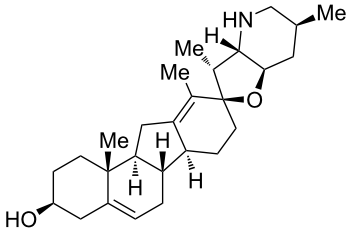
 **6**: bis-*exo*-cyclopamine	8 steps from **3**	18%	0.2 ± 0.01 μM	stable
 **8**	4 steps from **3**	27%	> 10 μM	stable
 **9**	5 steps from **3**	35%	> 10 μM	stable
 **19**: 20-demethyl-bis-*exo*-cyclopamine	8 steps from **14**	7%	> 10 μM	decomp.
 **23**: F-*nor*-20,25-bis-demethyl-*exo*-cyclopamine	7 steps from **14**	9%	6.40 ± 0.90 μM	decomp.
Previously synthesized and biologically evaluated:				
 **2**: *exo*-cyclopamine	9 steps from **3**	7%	0.5 μM [34]	stable

Table 1: Synthesized *exo*-cyclopamine derivatives, number of steps, yields, results of their biological testing, and stability towards acid. (continued)

 **1**: cyclopamine	10 steps from **3**	5%	5 μM [34]	decomp.

bioactivity and acid stability was revealed. Our designed analogs of cyclopamine are accessible in noticeably shorter and higher yielding synthetic routes than the parent compound, a fact that will further contribute to their usefulness in biological and medicinal studies.

Supporting Information

Supporting Information File 1

Experimental details and analytical data of all synthesized compounds are provided.
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-9-267-S1.pdf]

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