Tetrahedron 67 (2011) 10261–10266

Contents lists available at SciVerse ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Design, synthesis, and biological evaluation of estrone-derived hedgehog signaling inhibitors

Jeffrey D. Winkler [a,*], André K. Isaacs [a], Chaomei Xiang [b], Valérie Baubet [b], Nadia Dahmane [b,*]

[a] Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104, USA
[b] The Wistar Institute, Philadelphia, PA 19104, USA

ARTICLE INFO

Article history:
Received 16 August 2011
Received in revised form 9 October 2011
Accepted 10 October 2011
Available online 19 October 2011

Dedicated with both great affection and admiration to Professor Gilbert Stork, a remarkable scientist and mentor who continues to inspire us all

Keywords:
Cyclopamine
Steroidal alkaloid
Cancer chemotherapy
Hedgehog signaling
Structure–activity relationships

ABSTRACT

The design, synthesis, and biological evaluation of new analogs of the naturally occurring compound cyclopamine, a hedgehog signaling inhibitor, are described. Structure–activity relationship studies lead to an evolving model for the pharmacophore of this medically promising compound class of anti-cancer chemotherapeutic agents.

1. Introduction

More than 40 years ago, Binns and co-workers established that the alkaloid cyclopamine **1** (Fig. 1a) was responsible for the birth defects observed in calves from livestock that were fed diets rich in the corn lily, *Veratrum californicum* (Fig. 1b).[1,2] The observed phenotype included anophthalmia, cyclopia, and severe craniofacial effects (Fig. 1c).

It was later established that cyclopamine **1** acts by inhibiting the Sonic Hedgehog (SHH) cellular signaling pathway, which is critical for tissue growth and differentiation, thus playing a pivotal role in embryogenesis.[3,4] Activation of the SHH-signal transduction pathway is initiated by the binding of the SHH ligand to the cellular membrane receptor Patched (PTCH1), which relieves the PTCH1-mediated inhibition of the transmembrane protein Smoothened (SMO) (Fig. 1d).[5,6] Activated SMO transduces the signal to the nucleus to regulate gene expression via Gli transcription factors. Beachy and co-workers have established that **1** disrupts this pathway by inhibition of SMO.[7]

SHH signaling was first linked to cancer with the identification of mutations in the *PTCH1* gene in Gorlin syndrome patients.[8] It was subsequently shown that this pathway is also active in the majority of sporadic basal cell carcinomas.[9] In addition, activation of the SHH pathway has been linked to brain tumors, including medulloblastomas and gliomas,[12] melanoma,[13] lung adenocarcinoma,[14] as well as prostate,[15] small cell lung,[16] and pancreatic cancer.[17] Treatment of cancer cells with cyclopamine **1** induces a decrease in proliferation, an increase of apoptosis and/or a decrease of metastasis.[10,18] The teratogenicity associated with cyclopamine has not hampered interest in this natural product as an important lead structure in the development of cancer chemotherapeutic agents that act via inhibition of SHH signaling.[19]

In spite of the attractive pharmacological profile against a number of cancer xenografts, in vivo evaluation of cyclopamine has been hampered by its poor aqueous solubility (ca. 5 mg/ml) and acid lability. Under acidic conditions, cyclopamine **1** readily converts to veratramine **2**, via cleavage of the spirotetrahydrofuran ring, followed by aromatization of the D ring.[20] Unlike cyclopamine, **2** does not act as an SHH antagonist, and causes hemolysis by targeting other receptors[21] (Fig. 2).

Two strategies have been reported to address the issues of water solubility and acid lability of **1**: (1) the covalent modification of **1** to

* Corresponding authors. E-mail address: winkler@sas.upenn.edu (J.D. Winkler).

0040-4020/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2011.10.028

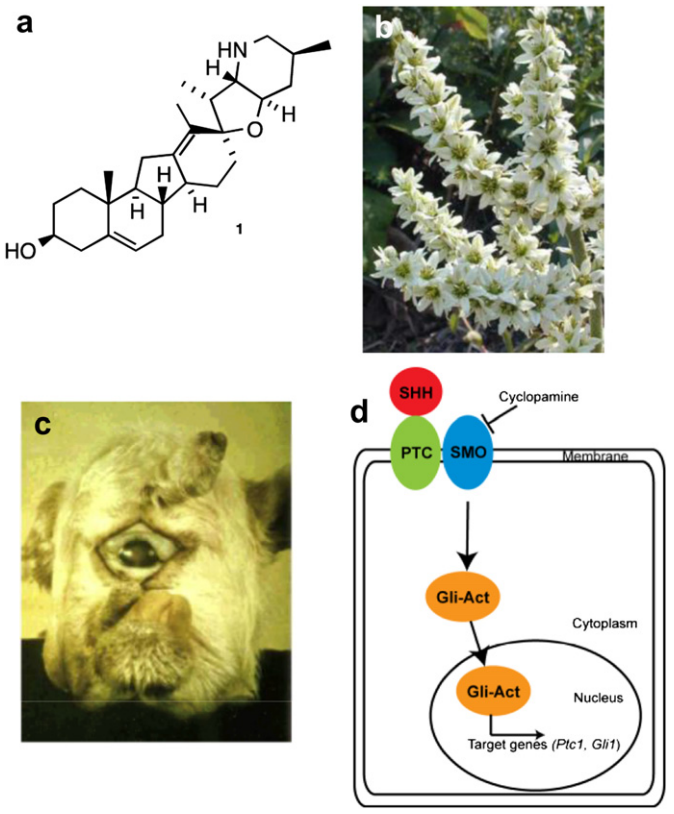


Fig. 1. (a) Structure of cyclopamine **1**; (b) *V. californicum*;[10] (c) cyclopia;[11] and (d) a simplified schematic of SHH signaling showing the effect of **1** on SMO.

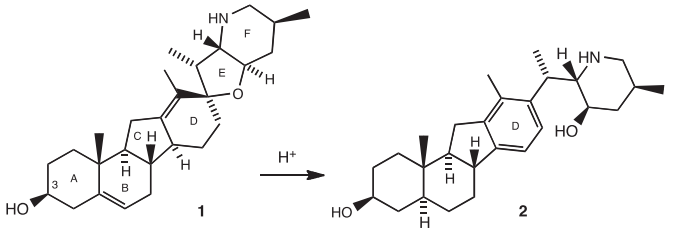


Fig. 2. Conversion of cyclopamine **1** to the D-ring aromatic compound veratramine **2** under acidic conditions.

produce structurally related and metabolically stable lead structures, i.e., IPI-926 **3**,[22] in which the D ring of **1** is expanded to a seven-membered ring, as pioneered by Tremblay and co-workers at Infinity Pharmaceuticals; and (2) the screening of libraries of diverse chemical structures in the hope of discovering drug-like structures that will interfere with SHH signaling.[23] The most noteworthy success to date using this approach is GDC-0449 **4** (Fig. 3), a compound that is currently in Phase II clinical trials.[24] The first approach, however, relies on the availability of the natural product **1**, which is expensive, and recent results indicate an acquired resistance to inhibition of SHH signaling in an MB patient treated with the GDC-0449, providing the impetus for the development of new SHH signaling antagonists.[25]

Consequently, there is an urgent need to identify readily available potent inhibitors of SHH as lead structures for the development of new cancer chemotherapies. We report herein the design and synthesis of cyclopamine-like structures derived from readily available steroidal precursors that function as potent cyclopamine mimetics. Outlined herein are the results of our structure–activity relationship studies on this novel compound class.

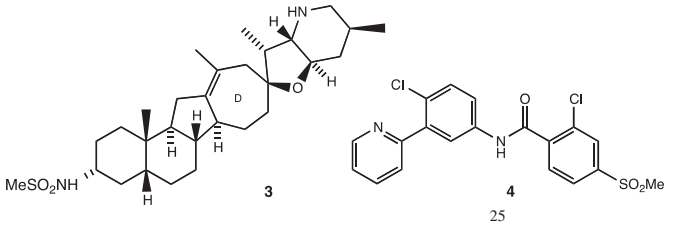


Fig. 3. Structures of hedgehog signaling inhibitors IPI-926 (Infinity Pharmaceuticals) **3** and GDC-0449 (Curis/Genentech) **4**.

2. Results and discussion

We have opted to explore a third approach to the identification of novel SHH signaling inhibitors, which is not dependent on the availability of **1**, and yet generates new lead compounds that closely resemble **1** in both structure and function. The difference in teratogenicity between cyclopamine **1** and the close structural analog tomatidine **5** (Fig. 4; non-teratogenic) has been attributed to the difference in the orientation of the nitrogen atom relative to the steroid plane in **1** and **5**. The C-nor-D-homo framework of **1** can thus be viewed as a scaffold that orients the E/F heterobicyclic moiety orthogonal to the steroidal ring system, with the F-ring nitrogen atom on the α-face of the steroid plane.[26] In contrast, the tetrahydrofuran ring of **5** lies in the steroid plane and the nitrogen atom is on the β-face of the steroid plane.

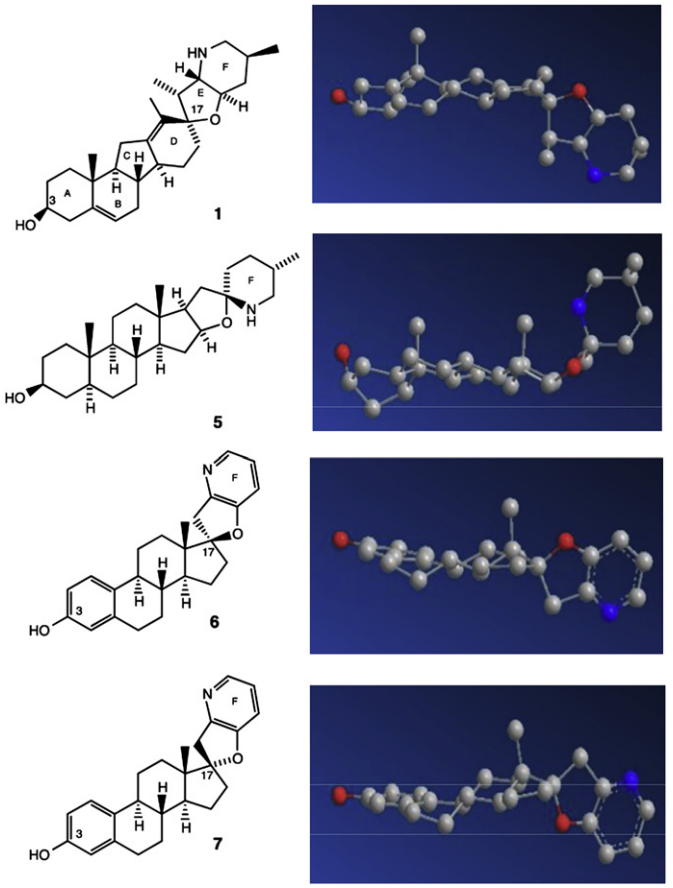


Fig. 4. Structures and energy-minimized structures of cyclopamine **1**, tomatidine **5**, estrone-derived analog **6**, and **7**, the C-17 epimer of **6**.[27]

We reasoned that the C-nor-D-homo steroidal ring system of **1** functions as a scaffold for the orientation of the heterobicyclic framework of the EF rings relative to the C-3β oxygen functionality in **1**. Replacement of the C-nor-D-homo steroidal system with the

androstane ring system and further stereochemical simplification via aromatization of rings A and F leads to the novel estrone-derived analog **6**.[27]

The energy-minimized structures in Fig. 4 suggest an important role for the C-17 stereochemistry common to both **1** and **3**, which, unlike **5** (and **7**, the C-17 *epi* analog of **6**), share the orientation of the C-17 oxygen substituent on the β-face of the steroid plane. In contrast, the C-17 oxygen atom of **7**, the C-17 epimer of **6**, is oriented on the α-face of the steroid plane, which leads to the orientation of the F-ring nitrogen atom of **7** on the β-face of the steroid plane, the same orientation that is found in tomatidine **5**, a naturally occurring compound, which displays no activity as a hedgehog signaling inhibitor.

2.1. Synthesis and biological evaluation of estrone-derived analogs 6, 7, and 8 of cyclopamine 1

To test the hypothesis that the three-point recognition of the C-3 oxygen, C-17 oxygen, and C-21 nitrogen heteroatoms as oriented in **1** is required for recognition at SMO, we have synthesized both **6**[27] and **7**.[28] As previously described, both **6** and **7** are potent inhibitors of SHH signaling as evaluated by inhibition of ligand-induced SHH signaling activity in a luciferase-based assay and by inhibition of SHH-induced proliferation of mouse granule neuron precursors, with activities comparable or superior to that of cyclopamine **1** at concentrations as low as 5 μM.

These results, the comparable potency of **6** and the C-17 epimer **7**, challenged our hypothesis that the three-point recognition of the C-3 oxygen and each of the other E and F-ring heteroatom functionalities in **1** (and **6**) is required for recognition at SMO, the cellular target of cyclopamine, since structures with either orientation at C-17, i.e., both **6** and **7**, are potent inhibitors of SHH signaling. The relative orientations of the tetrahydrofuran oxygen and pyridine nitrogen relative to the steroid plane do not appear to be important features for recognition of these cyclopamine analogs at SMO, suggesting that the C-3 oxygen functionality may not be required for recognition at SMO.

To establish the role, if any, of the C-3 oxygen functionality that is present in **6** and **7** on the biological activity of these estrone-derived analogs of cyclopamine **1**, we prepared the C-3 deoxy compound **8** (Fig. 5) as previously described.[28] Biological evaluation of **8** using the same GLI-luciferase assay[29] described for **6** and **7** revealed that **8** is a potent inhibitor of SHH signaling. In this assay, the C-3 deoxy analog **8** led to a strong inhibition of SHH signaling activity (80% inhibition at 5 μM; compared to 70% inhibition in the same assay with **6**). We have also reported that the C-3 deoxy analog **8** is ca. 2× more potent than cyclopamine **1** at reducing DAOY medulloblastoma cell viability, an important measure of SHH inhibitory activity, and a significant illustration of the potential of these structures for the development of brain cancer chemotherapeutics.[28]

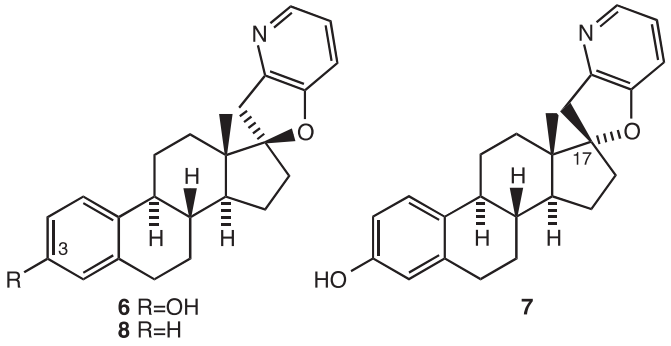
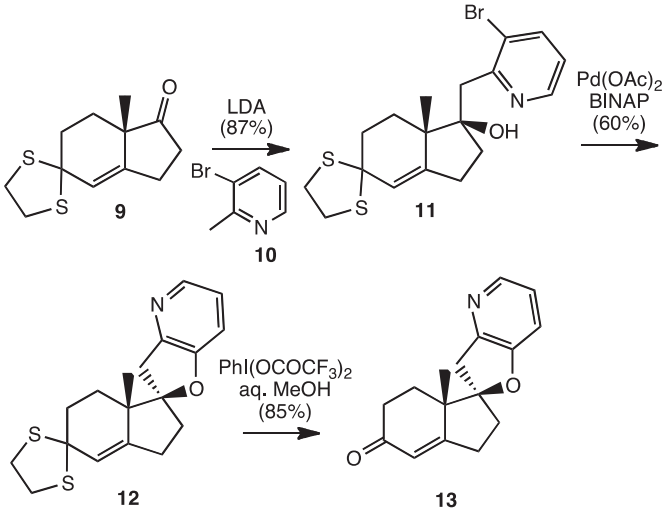


Fig. 5. Structures of estrone-derived cyclopamine analog **6**, the C-17 *epi* analog **7**, and the C-3 deoxy analog **8**.

2.2. Synthesis and biological evaluation of truncated analogs of cyclopamine 1

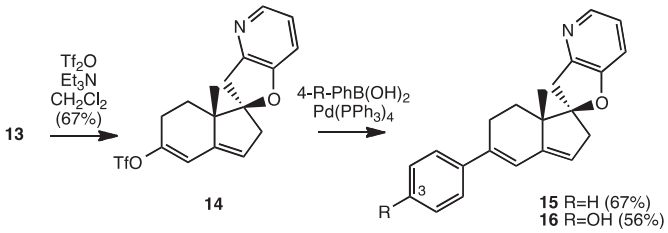
These results necessitate a revision of the binding model that we originally advanced (Fig. 4).[27,28] The potent activity of **8** suggests that the C-3 hydroxyl common to both **6** and **7** is not required for biological activity, and brings into question the importance of the intact steroidal framework. To examine the effect of truncating the steroid, we have examined the deletion of portions of the tetracyclic steroidal ring system common to **6**, **7**, and **8**.

Toward that end, we have prepared **13** (Scheme 1), an analog lacking the AB ring system present in **6**, **7**, and **8**. Using the same annelation strategy that was employed for the syntheses of the previously described analogs, addition of the conjugate base of **10** to the known thioketal **9**[30] led to the formation of carbinol **11**, which on Buchwald–Hartwig cyclization generated **12**, containing the dihydrofuropyridine that constitutes the EF ring system of cyclopamine **1**. Dithioketal deprotection of **12** gave the desired truncated analog **13** in good yield.



Scheme 1. Syntheses of des-AB analog **13**.

Biological evaluation of **13** using the same SHH-Light2 cells luciferase-based assay[29] that was used to evaluate the biological activity of **6**, **7**, and **8** reveals that the tetracyclic analog **20**, lacking the steroidal A and B rings contained in all of the previously described analogs, has no effect on SHH signaling, and suggests that the AB ring system is important for SHH signaling inhibitory activity. This finding prompted us to examine the preparation of analogs that would more closely resemble the structures of the previously prepared estrone-based systems, i.e., containing the aromatic A ring that is present in **6**, **7**, and **8**. The synthesis of des-B (lacking the steroidal B ring common to **6**, **7**, and **8**) analogs **15** and **16** is outlined in Scheme 2.



Scheme 2. Syntheses of des-B analogs **15** and **16**.

Reaction of the dienol triflate **14**, derived from **13**, via Suzuki coupling of **14** with both phenylboronic acid and *p*-hydroxy-phenylboronic acid, respectively, provided **15** and **16**, both of which lack the B-ring present in cyclopamine. Based on the observation that the C-3 deoxy analog **8** (Fig. 5) was more potent than the C-3 hydroxy compound **6** (and **7**),[28] we were surprised to find that **16** (R=OH) is more potent than **15** (lacking the C-3 hydroxy group) in the previously described luciferase-based assay for hedgehog signaling activity, as shown in Fig. 6.

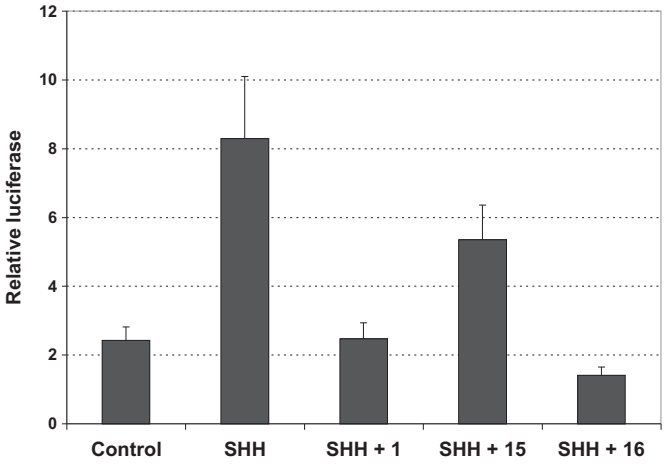


Fig. 6. Luciferase-based assay for SHH activity: treatment of SHH-Light2 cells with recombinant SHH (200 ng) resulted in the strong induction of reporter activity, which was blocked by co-treatment with either cyclopamine **1** or with **15** or **16** at 5 μM, both $P<0.001$ [SHH vs SHH+**1**; SHH vs SHH+**15**/**16**].

Further investigation with **16** revealed that it is a potent SHH signaling inhibitory compound, as demonstrated in the GNP proliferation assay as shown in Fig. 7, where it is ca. 3× more potent than cyclopamine **1**. We have also established that **16** is ca. equipotent with cyclopamine **1** in the DAOY medulloblastoma cell viability assay, as illustrated in Fig. 8.

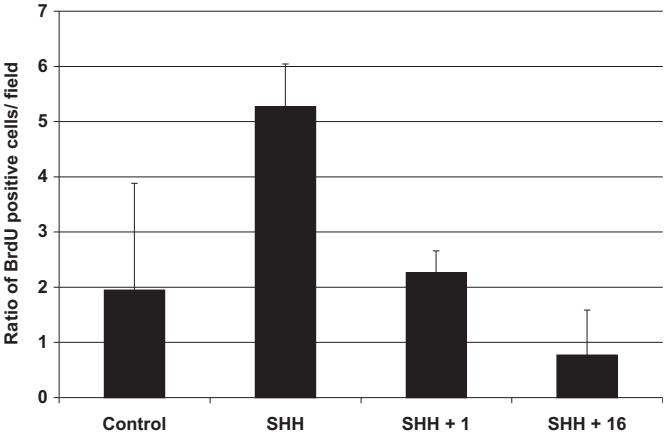


Fig. 7. Estrone-derived analog **16** inhibits SHH-induced proliferation of granule neuron precursors (GNPs).

3. Conclusions

We have demonstrated that it is possible to replace the C-nor-D-homo ring system of cyclopamine **1** with an estrone-derived steroidal ring system and to prepare a cyclopamine analog **6** that is a potent SHH signaling inhibitor as measured in both the luciferase and GNP (granule neuron precursor) assays.[27]

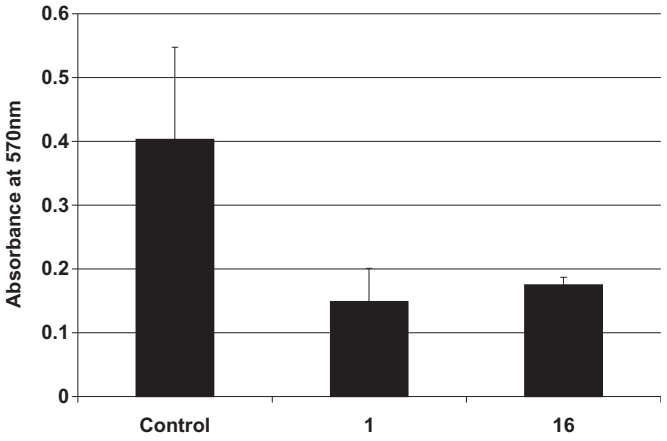


Fig. 8. Analog **16** reduces DAOY medulloblastoma cell viability. DAOY human medulloblastoma cells were treated with either carrier DMSO (control), cyclopamine **1** (10 μM) or **16** (10 μM) for 3 days. The histogram measures cell viability assessed by the MTT assay (absorbance at 570 nm) (asterisk indicates $p<0.05$). Similar results were obtained with U87GBM cells (not shown).

Evaluation of the SAR of this lead compound by examination of the C-17 *epi* compound **7** and the C-3 deoxy analog **8** established that the two-point binding model (using the C-3β hydroxyl and the heterobicyclic EF ring system of **1** (and **6**)) at the cellular receptor SMO is not sufficient to explain the surprising level of potency observed for **7** and **8**.[28]

This important finding led us to examine the synthesis and biological evaluation of truncated structures, such as **13**, lacking both the A and B rings common to the previously described structures. We have found such a structural modification too extreme to retain SHH inhibitory activity, but we report that the addition of the aromatic A ring to **13** leads to potent SHH signaling inhibitors.

The introduction of the aromatic A ring, that is present in **6**, **7**, and **18**, leads to novel des-B structures **15** and **16**, i.e., lacking the steroidal B ring. Biological evaluation of **15** and **16** reveals that, in contrast to **6** (C-3 hydroxy) and **8** (C-3 deoxy), in which removal of the C-3 hydroxyl leads to more potent inhibitory activity, the C-3 hydroxylated analog **16** is decidedly more potent than the C-3 deoxy compound **15**. The basis for this difference is currently under investigation in our laboratories. Biological evaluation of **16** establishes that it is more potent than cyclopamine **1** in the inhibition of SHH-induced proliferation of GNPs (Fig. 7) and ca. equipotent with **1** in the DAOY medulloblastoma cell viability assay (Fig. 8).

These findings suggest that partial structures of estrone-based analogs of **1** are sufficient to generate potent SHH signaling inhibitors. Further work on the development of more potent compounds is currently underway in our laboratory and our results will be reported in due course.

4. Experimental section

4.1. General methods

Solvents used for extraction and purification were HPLC grade from Fisher. Unless otherwise indicated, all reactions were run under an inert atmosphere of Argon. Anhydrous tetrahydrofuran, ethyl ether, and toluene were obtained via passage through an activated alumina column.[31] Commercial reagents were used as received. Deuterated solvents were obtained from Cambridge Isotope labs. Merck pre-coated silica gel plates (250 μm, 60 F_{254}) were used for analytical TLC. Spots were visualized using 254 nm ultraviolet light, with either anisaldehyde or potassium permanganate stains as visualizing agents. Chromatographic purifications were performed on Sorbent Technologies silica gel (particle size 32–63 μ). ^{1}H and ^{13}C

NMR spectra were recorded at 500 MHz and 125 MHz, respectively, in $CDCl_3$ on a Bruker AM-500 or DRX-500 spectrometer. Chemical shifts are reported relative to internal chloroform (δ 7.26 for ^1H, δ 77.0 for ^{13}C). Infrared spectra were recorded on a NaCl plate using a Perkin–Elmer 1600 series Fourier transform spectrometer. High resolution mass spectra were obtained at the University of Pennsylvania Mass Spectrometry Service Center on an Autospec high resolution double-focusing electrospray ionization/chemical ionization spectrometer with either DEC 11/73 or OPUS software data system. Melting points were obtained on a Thomas Hoover capillary melting point apparatus and are uncorrected.

*4.1.1. (1′R,7a′S)-1′-((3-Bromopyridin-2-yl)methyl)-7a′-methyl-1′,2′,3′,6′,7′,7a′-hexahydrospiro[[1,3]dithiolane-2,5′-inden]-1′-ol (**11**).* To a solution of diisopropylamine (2.6 mL, 18.6 mmol) in dry Et_2O (6 mL) stirred at 0 °C under argon was added dropwise a solution of 2.5 M *n*-BuLi in hexanes (7.4 mL, 18.6 mmol). The solution was stirred at 0 °C for 30 min. The flask was cooled to −20 °C and a solution of 2-methyl-3-bromopyridine (3.19 g, 18.6 mmol) in Et_2O (9 mL) was added dropwise. The resulting red mixture was stirred at −20 °C, under argon, for 2 h. A solution of thiolane **9** (1.78 g, 7.42 mmol) in THF (11 mL) was added dropwise and kept stirring at −20 °C for 1 h. The reaction flask was allowed to warm up to room temperature and was quenched slowly with H_2O (30 mL). The mixture was extracted with EtOAc (3×75 mL), washed with saturated NH_4Cl (30 mL), saturated $NaHCO_3$ (30 mL), brine (30 mL), and dried with Na_2SO_4. The solvent was removed under reduced pressure and the crude product was purified by silica gel chromatography (10% ethyl acetate in hexanes) to yield **11** as an off-white solid (2.66 g, 87%, mp 71–73 °C). $[\alpha]_D^{21.2}$ −91.9 (*c* 1.0, $CHCl_3$). ^1H NMR ($CDCl_3$): δ=8.41 (dd, *J*=1.5, 4.5 Hz, 1H), 7.88 (dd, *J*=1.5, 8.0 Hz, 1H), 7.06 (dd, *J*=4.5, 8.0 Hz, 1H), 6.31 (s, 1H), 5.58 (s, 1H), 3.43–3.35 (m, 3H), 3.25–3.19 (m, 1H), 3.11 (d, *J*=15.5 Hz, 1H), 2.98 (d, *J*=15.5 Hz, 1H), 2.50–2.44 (m, 1H), 2.34 (m, 1H), 2.26–2.17 (m, 2H), 1.89–1.82 (m, 1H), 1.77 (m, 1H), 1.61–1.58 (m, 1H), 1.48 (m, 1H), 1.18 (s, 3H). ^{13}C NMR ($CDCl_3$): δ=159.3, 147.4, 146.6, 140.8, 124.4, 122.7, 122.6, 82.7, 66.0, 47.1, 40.4, 40.2, 39.7, 38.9, 33.5, 29.6, 26.0, 19.8. FTIR (thin film) 3362, 2922, 1428, 1066, 1033 cm^{-1}. HRMS (ES) calcd for $C_{18}H_{22}BrNOS_2$: 411.0326 (M^+), found 412.0388 (MH^+).

*4.1.2. (1′R,7a′S)-7a′-Methyl-2′,3′,7′,7a′-tetrahydro-3H-spiro[furo[3,2-b]pyridine-2,1′-inden]-5′(6′H)-one (**13**).* A resealable Schlenk tube was charged with alcohol **11** (90 mg, 0.2 mmol), $Pd(OAc)_2$ (10 mg, 0.04 mmol), BINAP (27 mg, 0.04 mmol) and Cs_2CO_3 (107 mg, 0.3 mmol). Dry toluene (3 mL) was added and the tube was capped under argon and the resulting mixture was allowed to stir at 80 °C for 3 h. The mixture was allowed to cool to room temperature, filtered through Celite, concentrated under reduced pressure and purified by silica gel chromatography (15% ethyl acetate in hexanes) to yield **12** as a white solid (43 mg, 60%): mp 134–136 °C. $[\alpha]_D^{19.2}$ −29.7 (*c* 1.0, $CHCl_3$). ^1H NMR ($CDCl_3$): δ=8.02 (dd, *J*=2.0, 4.0 Hz, 1H), 6.98 (m, 2H), 5.63 (s, 1H), 3.42–3.33 (m, 4H), 3.22–3.16 (m, 1H), 2.90 (d, *J*=17.0 Hz, 1H), 2.60–2.54 (m, 1H), 2.45–2.39 (m, 1H), 2.31–2.15 (m, 3H), 1.95–1.91 (m, 1H), 1.75 (td, *J*=3.5, 13.5 Hz, 1H), 1.33 (dt, *J*=3.5, 13.5 Hz, 1H), 1.22 (s, 3H). ^{13}C NMR ($CDCl_3$): δ=153.5, 150.8, 144.0, 141.4, 125.8, 122.2, 114.9, 96.1, 65.3, 46.4, 40.5, 40.0, 39.7, 38.3, 34.7, 29.2, 24.9, 19.8. FTIR (thin film) 2923, 1429, 1001 cm^{-1}. HRMS (ES) calcd for $C_{18}H_{21}NOS_2$: 331.1064 (M^+), found 332.1129 (MH^+).

To a solution of thioketal **12** (35 mg, 0.11 mmol) in MeOH (1.4 mL), H_2O (0.2 mL) and CH_2Cl_2 (0.7 mL) was added THF (0.1 mL) followed by bis(trifluoroacetoxy)iodobenzene (68 mg, 0.16 mmol) at room temperature. After 10 min, the solution was poured into a saturated $NaHCO_3$ solution (5 mL) and extracted with CH_2Cl_2 (3×10 mL). The combined organic layers were washed with brine (10 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by silica gel chromatography (50% ethyl acetate in hexanes) yielded **13** as a white solid (23 mg, 85%): mp 133–135 °C. $[\alpha]_D^{19.6}$ −56.4 (*c* 1.0, $CHCl_3$). ^1H NMR ($CDCl_3$): δ=8.04 (s, 1H), 7.02 (m, 2H), 5.88 (s, 1H), 3.41 (d, *J*=17.0 Hz, 1H), 2.98 (d, *J*=17.0 Hz, 1H), 2.82 (m, 1H), 2.53 (m, 3H), 2.37 (dd, *J*=5.0, 13.0 Hz, 1H), 2.05 (m, 2H), 1.63 (m, 1H), 1.40 (s, 3H). ^{13}C NMR ($CDCl_3$): δ=198.1, 172.1, 153.2, 150.0, 141.9, 124.3, 122.6, 115.3, 95.7, 47.9, 39.1, 34.7, 33.0, 28.9, 26.3, 18.7. FTIR (thin film) 3428, 2930, 1666, 1430, 1258, 1004 cm^{-1}. HRMS (ES) calcd for $C_{18}H_{17}NO_2$: 255.1259 (M^+), found 256.1332 (MH^+).

*4.1.3. (1′R,7a′S)-7a′-Methyl-2′,6′,7′,7a′-tetrahydro-3H-spiro[furo[3,2-b]pyridine-2,1′-inden]-5′-yl trifluoromethanesulfonate (**14**).* A solution of **13** (64 mg, 0.25 mmol) in dry CH_2Cl_2 (1.2 mL) was cooled to −20 °C and triethylamine (42 μL, 0.30 mmol) was added dropwise to the stirring solution. After a period of 5 min, trifluoromethanesulfonic anhydride (50 μL, 0.30 mmol) was added dropwise and the solution was allowed to warm up to 0 °C over a 1 h period. The reaction mixture was diluted with CH_2Cl_2 (10 mL) and quenched with brine (5 mL). The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (10 mL). The combined organic layers were dried with Na_2SO_4 and the solvent was removed under reduced pressure. Purification by silica gel chromatography (33% ethyl acetate in hexanes) gave **14** as an orange oil (65 mg, 67%). ^1H NMR ($CDCl_3$): δ=8.03 (m, 1H), 7.02 (m, 2H), 6.23 (*J*=3.0 Hz, 1H), 5.64 (s, 1H), 3.51 (d, *J*=16.5 Hz, 1H), 3.08 (d, *J*=17.0 Hz, 1H), 2.96 (d, *J*=17.0 Hz, 1H), 2.64 (dd, *J*=3.0, 17.0 Hz, 2H), 2.43 (dd, *J*=5.5, 18.0 Hz, 1H), 1.89 (dt, *J*=5.5, 12.0 Hz, 1H), 1.54 (dd, *J*=5.5, 12.0 Hz, 1H), 1.19 (s, 3H). ^{13}C NMR ($CDCl_3$): δ=153.1, 150.7, 150.6, 141.9, 141.7, 123.7, 122.5, 115.4, 115.1, 97.1, 47.1, 44.6, 39.2, 27.6, 26.1, 17.6. FTIR (thin film) 2935, 1423, 1212 cm^{-1}. HRMS (ES) calcd for $C_{17}H_{16}F_3NO_4S$: 387.0752 (M^+), found 388.0821(MH^+).

*4.1.4. (1′R,7a′S)-7a′-Methyl-5′-phenyl-2′,6′,7′,7a′-tetrahydro-3H-spiro[furo[3,2-b]pyridine-2,1′-indene] (**15**).* To a solution of triflate **14** (16 mg, 0.04 mmol) in a 600 μL of a 1:1 THF/$PhCH_3$ mixture were added phenylboronic acid (5 mg, 0.04 mmol), $Pd(PPh_3)_4$ (2.4 mg, 0.002 mmol) followed by a 0.5 N solution of Na_2CO_3 (80 μL, 0.04 mmol). The mixture was heated to reflux for 3 h. The reaction flask was allowed to cool to room temperature and then diluted with H_2O (5 mL). The mixture was partitioned with CH_2Cl_2 (3×10 mL) and the combined organic layers were washed with brine (10 mL). The organic layer was dried with Na_2SO_4 and the solvent was removed under reduced pressure. Purification by silica gel chromatography (33% ethyl acetate in hexanes) yielded **15** as a yellow oil (12 mg, 92%). $[\alpha]_D^{23.6}$ −111.2 (*c* 0.36, $CHCl_3$). ^1H NMR ($CDCl_3$): δ=8.03 (t, *J*=2.8 Hz, 1H), 7.48 (d, *J*=8.5 Hz, 2H), 7.34 (t, *J*=7.8 Hz, 2H), 7.26 (m, 1H), 7.02 (m, 2H), 6.63 (s, 1H), 5.52 (s, 1H), 3.61 (d, *J*=17.0 Hz, 1H), 3.11 (d, *J*=16.5 Hz, 1H), 2.98 (d, *J*=17.0 Hz, 1H), 2.66 (m, 2H), 2.61 (dd, *J*=3.0, 16.5 Hz, 1H), 1.88 (m, 1H), 1.63 (m, 1H), 1.22 (s, 3H). ^{13}C NMR ($CDCl_3$): δ=153.4, 151.4, 146.0, 141.3, 140.8, 138.5, 128.4, 127.5, 125.2, 122.3, 120.0, 119.4, 115.2, 98.2, 47.0, 44.1, 39.2, 28.4, 25.6, 17.9. FTIR (thin film) 2928, 1429, 993 cm^{-1}. HRMS (ES) calcd for $C_{22}H_{21}NO$: 315.1623 (M^+), found 316.1719 (MH^+).

*4.1.5. 4-((1′R,7a′S)-7a′-Methyl-2′,6′,7′,7a′-tetrahydro-3H-spiro[furo[3,2-b]pyridine-2,1′-inden]-5′-yl)phenol (**16**).* To a solution of triflate **14** (19 mg, 0.05 mmol) in a 740 μL of a 1:1 THF/$PhCH_3$ mixture were added phenylboronic acid (7 mg, 0.05 mmol), $Pd(PPh_3)_4$ (3 mg, 0.003 mmol) followed by a 0.5 N solution of Na_2CO_3 (100 μL, 0.05 mmol). The mixture was heated to reflux for 3 h. The reaction flask was allowed to cool to room temperature and then diluted with H_2O (5 mL). The mixture was partitioned with CH_2Cl_2 (3×10 mL) and the combined organic layers were washed with brine (10 mL), dried with Na_2SO_4 and the solvent was removed under reduced pressure. Purification by silica gel chromatography (33% ethyl acetate in hexanes) yielded **16** as a yellow oil (9 mg, 56%). $[\alpha]_D^{23.9}$ −35.2 (*c* 0.64, $CHCl_3$). ^1H NMR ($CDCl_3$): δ=8.04 (t, *J*=3.0 Hz,

1H), 7.30 (d, J=8.5 Hz, 2H), 7.08 (d, J=3.0 Hz, 2H), 6.77 (d, J=8.5 Hz, 2H), 6.49 (s, 1H), 5.43 (s, 1H), 3.57 (d, J=17.0 Hz, 1H), 3.17 (d, J=16.5 Hz, 1H), 3.00 (d, J=17.0 Hz, 1H), 2.57 (dd, J=3.0, 16.5 Hz, 1H), 2.52 (m, 2H), 1.71 (m, 1H), 1.45 (dt, J=2.5, 12 Hz, 1H), 1.16 (s, 3H). ^{13}C NMR ($CDCl_3$): δ=156.5, 154.1, 151.2, 145.7, 140.4, 137.6, 132.3, 126.3, 122.7, 118.3, 117.8, 115.7, 115.6, 98.6, 47.5, 44.0, 39.2, 28.2, 25.3, 17.9. FTIR (thin film) 2926, 1513, 1433, 1278, 992 cm^{-1}. HRMS (ES) calcd for $C_{22}H_{21}NO_2$: 331.1572 (M^+), found 332.1661 (MH^+).

4.2. MTT cell viability assay

DAOY medulloblastoma or U87 glioma cells were plated in 96 well-plates at 3000 cells/well in DMEM/0.5% serum media. Cyclopamine or compounds to test were added at a concentration of 10 μM to the cells. As control, cells were treated with media containing DMSO only. Seventy-two hours later, cell viability was assayed using the MTT cell survival kit (Chemicon; cat# CT01) following the manufacturer protocol. For each assay, the measurement was done in triplicate. Each compound was tested at least in three independent experiments. A t-test was applied for statistical analysis.

4.3. Granule neuron progenitor proliferation assay

Granule neuron progenitors (GNPs) were purified from P5 mouse cerebella.[32] Cells were plated in 24-well plates with 800 000 cells/well and cultured in DMEM/F12 (Gibco, 11330), B27 (Gibco, 1X), N2 (Gibco, 1X), Glutamine (Cellgro, 2 mM), and Penicillin (50 units/ml)/Streptomycin (50 mg/ml) media. The day following plating, SHH (600 ng/ml, R&D) and/or compounds to test were added to the cells. After 24 h in culture with SHH and/or compounds, BrdU was added to the media at a final concentration of 12 mg/ml for 5 h. The cells were then rinsed with PBS and fixed on ice for 1 h and washed in PBS/0.1% TritonX-100 (PBT) before HCl treatment was carried out. Cells were treated with HCl 2 N for 30 min at 37 °C, then with 0.1 M of sodium borate, pH 8.5 for 20 min at room temperature and washed five times with PBT. Cells were incubated with a blocking solution (PBT with 10% goat serum) at room temperature for 1 h and then incubated with an anti-BrdU antibody (Becton Dickinson, 1:400). The cells were then washed and incubated with a secondary antibody anti-mouse FITC (Vector Lab, 1:500) for 1 h at room temperature. The nuclei were counterstained with Hoechst (Sigma) and then mounted in Mowiol/Dabco solution. BrdU-positive cells were counted with a fluorescence microscope using a 20× objective (Axioskop, Zeiss). At least five independent fields for each culture condition were counted. Statistical analysis was performed with the Student t test.

Acknowledgements

We gratefully acknowledge the generous financial support of the NIH (CA-134983) to J.D.W. and N.D.

Supplementary data

^{1}H and ^{13}C NMR spectra for all new compounds. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2011.10.028.

References and notes

1. Binns, W.; James, L. F.; Keeler, R. F.; Balls, L. D. *Cancer Res.* **1968**, *28*, 2323–2326.
2. Keeler, R. F.; Binns, W. *Can. J. Biochem.* **1966**, *44*, 829–838.
3. Cooper, M. K.; Porter, J. A.; Young, K. E.; Beachy, P. A. *Science* **1998**, *280*, 1603–1607.
4. Incardona, J. P.; Gaffield, W.; Kapur, R. P.; Roelink, H. *Development* **1998**, *125*, 3553–3562.
5. Hooper, J. E.; Scott, M. P. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 306–317.
6. Ingham, P. W.; McMahon, A. P. *Genes Dev.* **2001**, *15*, 3059–3087.
7. Chen, J. K.; Taipale, J.; Cooper, M. K.; Beachy, P. A. *Genes Dev.* **2002**, *16*, 2743–2748.
8. (a) Hahn, J.; Wicking, C.; Zaphiropoulous, P.; Gailini, M.; Shanley, S.; Chidambaram, A.; Vorechovsky, I.; Holmberg, E.; Unden, A.; Gillies, S.; Negus, K.; Smyth, I.; Pressman, C.; Leffell, D.; Gerrard, B.; Goldstein, A.; Dean, M.; Toftgard, R.; Chenevix-Trench, G.; Wainwright, B.; Bale, A. *Cell* **1996**, *85*, 841–851; (b) Johnson, R.; Rothman, A.; Xie, J.; Goodrich, L.; Bare, J.; Bonifas, J.; Quinn, A.; Myers, R.; Cox, D.; Epstein, E.; Scott, M. *Science* **1996**, *272*, 1668–1671.
9. Dahmane, N.; Lee, J.; Robbins, P.; Heller, P.; Ruiz i Altaba, A. *Nature* **1997**, *389*, 876–881.
10. http://www.dungevalley.co.uk.
11. http://externalities.tumblr.com/post/162704398/cyclopia.
12. (a) Berman, D.; Karhadkar, S.; Hallahan, A.; Pritchard, J.; Eberhart, C.; Watkins, D.; Chen, J.; Taipale, J.; Olson, J.; Beachy, P. *Science* **2002**, *297*, 1559–1561; (b) Clement, V.; Sanchez, P.; de Tribolet, N.; Radovanovic, I.; Ruiz i Altaba, A. *Curr. Biol.* **2007**, *17*, 165–172; (c) Dahmane, N.; Sanchez, P.; Gitton, Y.; Palma, V.; Sun, T.; Beyna, M.; Weiner, H.; Ruiz i Altaba, A. *Development* **2001**, *128*, 5201–5212.
13. Stecca, B.; Mas, C.; Clement, V.; Zbinden, M.; Correa, R.; Piguet, V.; Beermann, F.; Ruiz, A. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 5895–5900.
14. Yuan, Z.; Goetz, J.; Singh, S.; Ogden, S.; Petty, W.; Black, C.; Memoli, V.; Dmitrovsky, E.; Robbins, D. *Oncogene* **2007**, *26*, 1046–1055.
15. (a) Karhadkar, S.; Bova, G.; Abdallah, N.; Dhara, S.; Gardner, D.; Maitra, A.; Isaacs, J.; Berman, D.; Beachy, P. *Nature* **2004**, *431*, 707–712; (b) Sanchez, P.; Hernandez, A.; Stecca, B.; Kahler, A.; DeBueme, A.; Barrett, A.; Beyna, M.; Datta, M.; Datta, S.; Ruiz, A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 12561–12566; (c) Sheng, T.; Li, C.; Zhang, X.; Chi, S.; He, N.; Chen, K.; McCormick, F.; Gatalica, Z.; Xie, J. *Mol. Cancer* **2004**, *3*, 29.
16. Watkins, D.; Berman, D.; Burkholder, S.; Wang, B.; Beachy, P.; Baylin, S. *Nature* **2003**, *422*, 313–317.
17. Berman, D.; Karhadkar, S.; Maitra, A.; Montes, D.; Gerstenblith, M.; Briggs, K.; Parker, A.; Shimada, Y.; Eshleman, J.; Watkins, D.; Beachy, P. *Nature* **2003**, *425*, 846–851.
18. Sanchez, P.; Ruiz i Altaba, A. *Mech. Dev.* **2005**, *122*, 223–230.
19. For recent SAR on cyclopamine-derived structures, see (a) Heretsch, P.; Buttner, A.; Tzagkaroulaki, L.; Zahn, S.; Kirchner, B.; Giannis, A. *Chem. Commun.* **2011**, 7362–7364; (b) Tremblay, M.; Nevalainen, M.; Nair, S.; Porter, J.; Castro, A.; Behnke, M.; Yu, L.; Hagel, M.; White, K.; Faia, K.; Grenier, L.; Campbell, M.; Cushing, J.; Woodward, C.; Hoyt, J.; Foley, M.; Read, M.; Sydor, J.; Tong, J.; Palombella, V.; McGovern, K.; Adams, J. *J. Med. Chem.* **2008**, *51*, 6646–6649; For a general discussion, see Dolgin, E. *Nat. Med.* **2011**, *17*, 523.
20. (a) Keeler, R. F. *Teratology* **1970**, *3*, 169–173; (b) Wilson, S.; Strand, M.; Krapp, A.; Rise, F.; Petersen, D.; Krauss, S. *J. Pharm. Biomed. Anal.* **2010**, *52*, 707–713.
21. (a) Nagata, R.; Izumi, K. *Jpn. J. Pharmacol.* **1991**, *55*, 129–137; (b) Thron, C. D.; McCann, F. V. *Gen. Pharmacol.* **1998**, *32*, 81–89.
22. Tremblay, M.; Lescarbeau, A.; Grogan, M.; Tan, E.; Lin, G.; Austad, B.; Yu, L.; Behnke, M.; Nair, S.; Hagel, M.; White, K.; Conley, J.; Manna, J.; Alvarez-Diez, T.; Hoyt, J.; Woodward, C.; Sydor, J.; Pink, M.; MacDougall, J.; Campbell, M.; Cushing, J.; Ferguson, J.; Curtis, M.; McGovern, K.; Read, M.; Palombella, V.; Adams, J.; Castro, A. *J. Med. Chem.* **2009**, *52*, 4400–4418.
23. (a) Chen, J.; Taipale, J.; Young, K.; Maiti, T.; Beachy, P. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 14071–14076; (b) Frank-Kamenetsky, M.; Zhang, X.; Bottega, S.; Guicherit, O.; Wichterle, H.; Dudek, H.; Bumcrot, D.; Wang, F.; Jones, S.; Shulok, J.; Rubin, L.; Porter, J. *J. Biol.* **2002**, *1*, 10; (c) Williams, J.; Guicherit, O.; Zaharian, B.; Xu, Y.; Chai, L.; Wichterle, H.; Kon, C.; Gatchalian, C.; Porter, J.; Rubin, L.; Wang, F. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 4616–4621.
24. Sheridan, C. *Nat. Biotechnol.* **2009**, *27*, 968–969.
25. (a) Rudin, C.; Hann, C.; Laterra, J.; Yauch, R.; Callahan, C.; Fu, L.; Holcomb, T.; Stinson, J.; Gould, S.; Coleman, B.; LoRusso, P.; von Hoff, D.; de Sauvage, F.; Low, J. *N. Engl. J. Med.* **2009**, *361*, 1173–1178; (b) Yauch, R.; Dijkgraaf, G.; Alicke, B.; Januario, T.; Ahn, C.; Holcomb, T.; Pujara, K.; Stinson, J.; Callahan, C.; Tang, T.; Bazan, T.; Kan, Z.; Seshagiri, S.; Hann, C.; Gould, S.; Low, J.; Rudin, C.; de Sauvage, F. *Science* **2009**, *326*, 572–574.
26. Keeler, R. F. *Lipids* **1978**, *13*, 708–715.
27. For a preliminary account of the synthesis and biological evaluation of **6**, see Winkler, J.; Isaacs, A.; Holderbaum, L.; Tatard, V.; Dahmane, N. *Org. Lett.* **2009**, *11*, 2824–2827.
28. For a preliminary account of the synthesis and biological evaluation of **7** and **8**, see: Isaacs, A.; Xiang, C.; Baubet, V.; Dahmane, N.; Winkler, J. *Org. Lett.*, in press.
29. Taipale, J.; Chen, J. K.; Cooper, M. K.; Wang, B.; Mann, R. K.; Milenkovic, L.; Scott, M. P.; Beachy, P. A. *Nature* **2000**, *406*, 1005–1009.
30. Williams, J.; Sarkisia, G. *Synthesis* **1974**, 32–33.
31. Pangborn, A. B.; Giardello, M. A.; Grubbs, R. H.; Rosen, R. K.; Trimmers, F. J. *Organometallics* **1996**, *15*, 1518.
32. Fernandez, C.; Tatard, V.; Bertrand, N.; Dahmane, N. *Dev. Neurosci.* **2010**, *32*, 59–70.