Inhibitors

Deutsche Ausgabe: DOI: 10.1002/ange.201500112
Internationale Ausgabe: DOI: 10.1002/anie.201500112

Biology-Oriented Synthesis of a Withanolide-Inspired Compound Collection Reveals Novel Modulators of Hedgehog Signaling**

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Abstract: *Biology-oriented synthesis employs the structural information encoded in complex natural products to guide the synthesis of compound collections enriched in bioactivity. The trans-hydrindane dehydro-δ-lactone motif defines the characteristic scaffold of the steroid-like withanolides, a plant-derived natural product class with a diverse pattern of bioactivity. A withanolide-inspired compound collection was synthesized by making use of three key intermediates that contain this characteristic framework derivatized with different reactive functional groups. Biological evaluation of the compound collection in cell-based assays that monitored biological signal-transduction processes revealed a novel class of Hedgehog signaling inhibitors that target the protein Smoothened.*

Natural product structures remain a rich source of inspiration in the discovery and development of novel small-molecule modulators of bioactivity for chemical biology and medicinal chemistry research.[1] We have developed biology-oriented synthesis (BIOS) as a hypothesis-generating approach that employs the biological relevance encoded in natural products and their scaffold structures to guide the design and synthesis of compound collections enriched in diverse bioactivities.[2] Notably, BIOS can guide the simplification of natural-product structures while retaining the level of bioactivity, thereby addressing the synthesis and supply issues frequently raised as arguments against the use of natural products in medicinal chemistry.[3]

Herein, we report on the biology-oriented synthesis of a compound collection inspired by the withanolides, a structurally complex class of steroid-like natural products endowed with multiple bioactivities. Investigation of the compound collection for modulation of biological signal transduction revealed a novel class of Hedgehog signaling inhibitors that target the Smoothened protein.

The withanolides comprise a family of more than 300 plant-derived natural products (for representative examples, see Figure 1) possessing diverse bioactivities, which include

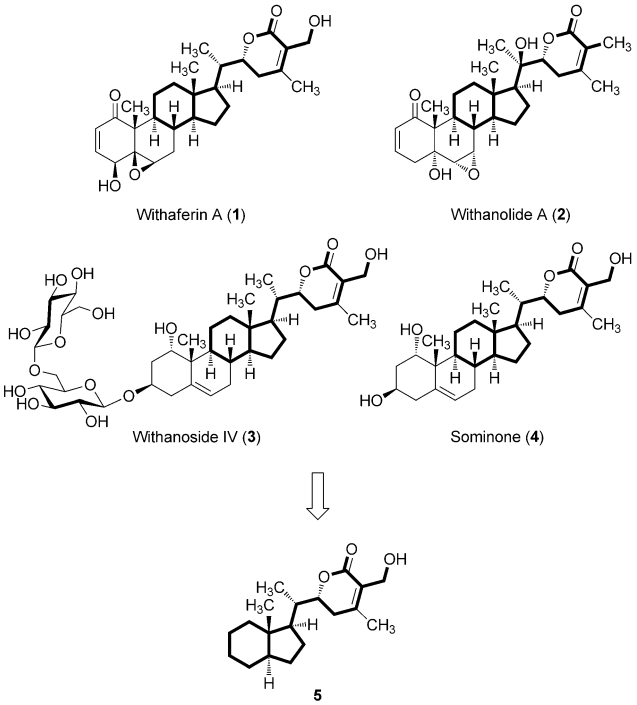

Figure 1. Structures of representative members of the withanolide natural-product family (**1**–**4**) and of the common *trans*-hydrindane dehydro-δ-lactone scaffold **5** (highlighted in bold).

potent anti-inflammatory effects,[4] the induction of neurite outgrowth and associated memory-enhancing effects[5] as well as the modulation of the mTOR[6] and Wnt pathways.[7] Notably, withanolide analogues are also bioactive.[8] A number of withanolides share a *trans*-hydrindane unit linked to an α,β-unsaturated lactone as a common structural denominator (Figure 1), and the withanolide structure primarily varies in the A and B rings of the steroid scaffold. Thus, we assumed that in general, the bioactivity is encoded in

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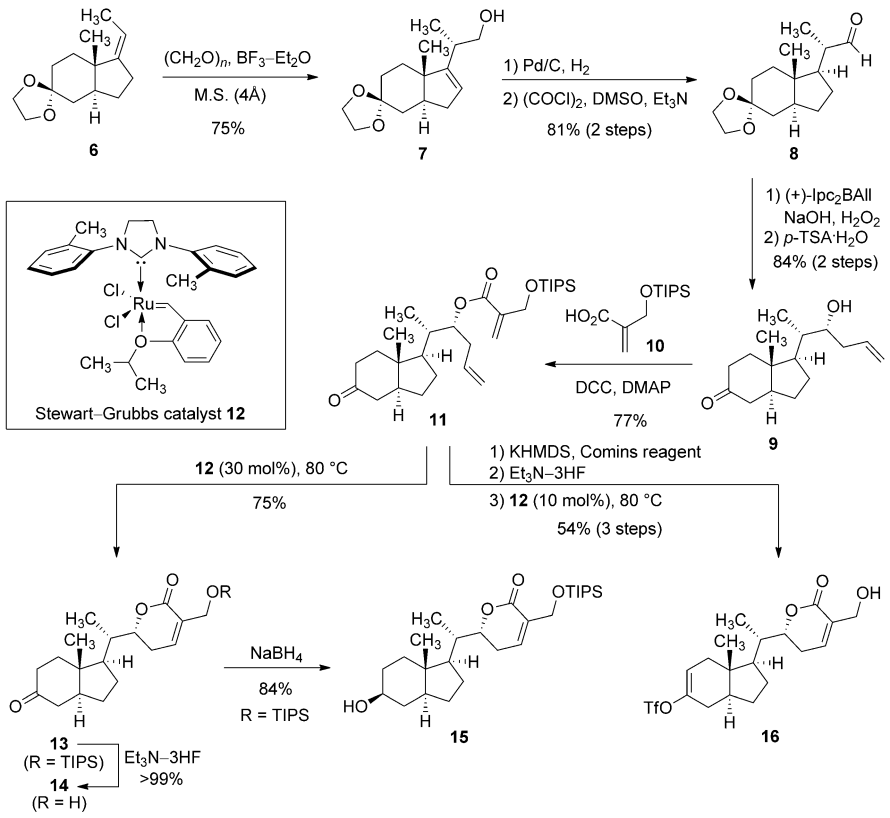
[**] This research was supported by the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013; ERC Grant 268309 to H.W.) and the Max Planck Society. J.S. and M.S. thank the Alexander von Humboldt Foundation and the Fonds der Chemischen Industrie for a post-doctoral and a Kekulé fellowship, respectively. L.M. thanks the European Regional Development Fund—Project FNUSA–ICRC (CZ.1.05/1.1.00/02.0123), the European Social Fund, and the State Budget of the Czech Republic.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201500112.

the *trans*-hydrindane/dehydro-δ-lactone part and is further modulated by different substituents. Consequently, a compound collection based on this scaffold should yield novel modulators of bioactivity.

The hydrindane-δ-lactone scaffold has not been explored in compound library development before. For the synthesis of such a collection, we envisaged to build up late-stage intermediates that could be transformed into a range of structurally simplified withanolide analogues in no more than two synthetic operations. δ-Lactones **13**–**16** meet this requirement (Scheme 1). For the synthesis of these intermediates, readily available *Z*-configured olefin **6** (prepared from the Hajos–Parrish ketone in multigram amounts)[9] was treated with paraformaldehyde and catalytic amounts of boron trifluoride etherate at 0 °C to yield homoallylic alcohol **7** in 75 % yield.[10] The use of activated 4 Å molecular sieves (M.S.) was required to prevent the otherwise facile cleavage of the acetal protecting group. Diastereoselective hydrogenation of the double bond,[11] followed by Swern oxidation, provided aldehyde **8**[12] in 81 % yield over two steps. The unstable aldehyde **8** was immediately subjected to an asymmetric allylation reaction using (+)-*B*-allyldiisopinocampheylborane.[12] Oxidative work-up followed by an acid-induced cleavage of the acetal protecting group yielded *R*-configured homoallylic alcohol **9** in 84 % yield over two steps. Esterification with acrylic acid derivative **10** gave acrylic ester **11** in 77 % yield.

Ring-closing olefin metathesis of protected acrylic ester **11** catalyzed by ruthenium(II) complex **12**[8a,13] followed by desilylation yielded δ-lactone **14** in 75 % yield (29 % over 8 steps from **6**). Chemo- and stereoselective[14] reduction of the ketone moiety in lactone **13** with excess sodium borohydride gave access to secondary alcohol **15** in 84 % yield (25 % over 8 steps from **6**). Alternatively, acrylate ester intermediate **11**[14] was treated with potassium hexamethyldisilazide, and the resulting enolate was trapped with Comins reagent[15] to give the corresponding enol triflate.[16] Cleavage of the triisopropylsilyl ether and ring-closing olefin metathesis of the unprotected α-hydroxymethyl acrylate catalyzed by ruthenium(II) complex **12**[13] provided the δ-lactone appended *trans*-hydrindane enol triflate **16** in 54 % yield over three steps (21 % over 9 steps from **6**).

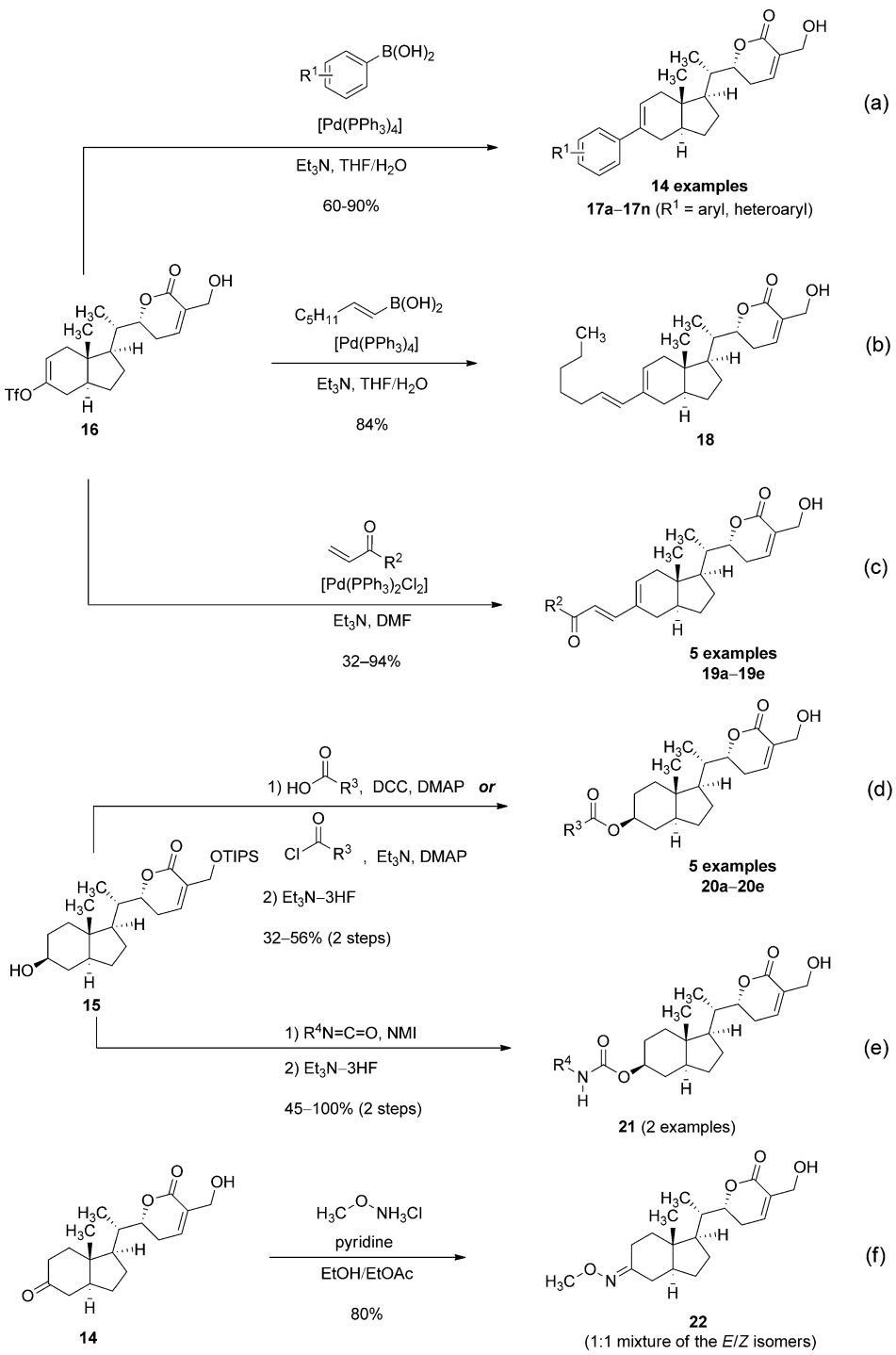


Scheme 1. Synthesis of *trans*-hydrindane dehydro-δ-lactone intermediates **13**–**16**. (+)-Ipc$_2$BAll = (+)-*B*-allyldiisopinocampheylborane, KHMDS = potassium hexamethyldisilazide, *p*-TSA = *para*-toluenesulfonic acid, Tf = trifluoromethanesulfonyl, TIPS = triisopropylsilyl.

To establish an initial compound collection, intermediates **14**–**16** were subjected to different transformations compatible with the dehydro-δ-lactone motif. Thus, enol triflate **16** underwent facile palladium-catalyzed cross-coupling reactions with aryl and alkenyl boronic acids and Heck reactions with electron-deficient olefins (Scheme 2 a–c). Functionalization of the secondary hydroxy group of monoprotected δ-lactone **15** and the keto group of unprotected δ-lactone **14** provided access to an alternative series of derivatives incorporating ester (**20**), carbamate (**21**), and oxime (**22**) groups (Scheme 2 d–f). Using the set of transformations illustrated in Scheme 2, a withanolide-inspired compound collection was assembled (see the Supporting Information for details).

The relative configurations of the members of the compound collection were assigned by comparison with the solved crystal structure of 4-acetylphenyl derivative **17a** (see the Supporting Information).[17] The crystal structure revealed that the configuration and the solid-state conformation of the dehydro-δ-lactone side chain resemble the reported crystal structures of natural withanolides.

To identify novel bioactive withanolide analogues, the compound collection was subjected to cell-based assays monitoring signal transduction pathways, which revealed four withanolide analogues that inhibited Hedgehog (Hh) signaling, that is, purmorphamine-induced osteogenesis in C3T/10T1/2 cells[18] (Table 1; see also the Supporting Information, Table S1). The products **17g**–**17h**, which were obtained by the Suzuki coupling reactions, as well as



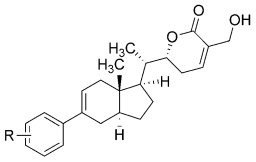
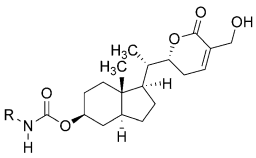
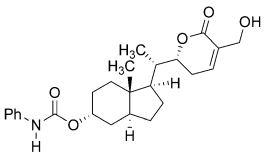
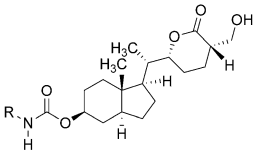
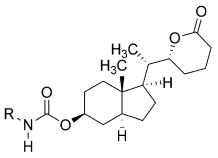
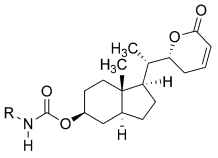
Scheme 2. Derivatization of *trans*-hydrindane dehydro-δ-lactone intermediates **14**–**16**. DCC = *N*,*N*′-dicyclohexylcarbodiimide, DMAP = 4-dimethylaminopyridine, NMI = *N*-methylimidazole.

carbamates **21a** and **21e** displayed comparable potency in this assay (Table 1). Among the two carbamates, *para*-chlorophenyl carbamate **21e** was found to be cytotoxic (Table 1). The Hh signaling pathway plays a fundamental role in embryonic and post-embryonic development, and aberrant Hh signaling has been linked to cancer.[19] Modulators of the Hh pathway are in clinical use, and novel inhibitor chemotypes are in high demand.[20] Binding of Hh to its transmembrane receptor Patched1 (Ptch1) relieves Ptch1-induced inhibition of the membrane protein Smoothened (Smo). Smo in turn activates the glioma-associated oncogene homologue (Gli) dependent transcription of Hh pathway-specific genes, such as Ptch1, Gli1, and Hip.[21] Consistent with inhibition of purmorphamine-induced osteogenesis, the carbamate withanolide analogue **21a** reduced the Gli-dependent reporter gene transcription in SHH-LIGHT2 cells with a half-maximal inhibitory concentration of $3.7 \pm 1.0\ \mu M$ (Figure 2a).[22] Furthermore, **21a** inhibited the expression of the Hedgehog target gene Ptch1 in NIH/3T3 cells upon stimulation with purmorphamine (Figure 2b).

The steroidal natural product cyclopamine[23] and oxy-steroids[24] (Figure S1) inhibit Hh signaling by targeting the Smo protein. Given the steroidal nature of the compound collection, we investigated whether the withanolide analogues also targeted the Smo protein. To this end, Smo-enriched membrane preparations were incubated with the [^{3}H]-labelled Smo antagonist cyclopamine, and competition by the four most active δ-lactones for binding to Smo was determined. To our delight, the Hh inhibitor **21a** competed with radiolabelled cyclopamine for binding to Smo with a K_i value of $57 \pm 10\ nM$ (Table 1).[25] In comparison, **21e** displayed a weaker Smo binding with a K_i value of $134 \pm 60\ nM$ (Table 1). Withanolide analogues obtained from the Suzuki coupling (**17g**–**h**) were significantly less potent (Table 1 and Table S1). Interestingly, the diastereoisomer **21a′** of the potent withanolide analogue **21**, which contains a urethane moiety in an α-configuration, was only moderately active. This result validates the design of the stereoselective *trans*-hydrindane dehydro-δ-lactone synthesis (Table 1).

To delineate a structure–activity relationship for the active Hh inhibitors, a series of urethane analogues were synthesized as described above for **21a**. To explore the

Table 1: Biological evaluation of withanolide analogues in Hedgehog pathway inhibition and Smoothened protein binding assays.

Withanolide analogues		IC_{50}[a] [μM] for osteogenesis inhibition	IC_{50}[b] [μM] (cell viability)	K_i value[c] for Smoothened binding [μM]
	**17g**; R = 3-NO_2	2.2 ± 0.7	inactive	6.1 ± 4.0
	17h; R = 2-OMe	1.9 ± 0.5	inactive	11.0 ± 3.0
	**21a**; R = Ph	1.8 ± 0.6	inactive	0.057 ± 0.01
	21e: R = 4-ClC_6H_4	2.3 ± 0.3	> 10	0.134 ± 0.06
	**21a′**	9.4 ± 2.8	inactive	n.d.
 (5 examples)	**24**	3.0–6.6[d]	inactive	n.d.
 (7 examples)	**27a**[e] R = Ph	6.0 ± 1.1	inactive	n.d.
 (6 examples)	**28**	2.7–7.9[d]	3.6–6.8	n.d.

[a] Mean IC_{50} values ± standard deviation ($n \geq 3$) for inhibition of the Hedgehog pathway as determined in an osteogenesis assay. [b] Influence on the viability of C3H10T1/2 cells as determined upon treatment with the compounds (10 μM) for 72 hours using the CellTiter-Glo assay. "> 10": cell viability at 10 μM: 50–70%; "inactive": cell viability at 10 μM: > 70% cells. [c] K_i values ± standard deviation ($n \geq 2$) as determined in a competition assay with the protein Smoothened using [^{3}H]-labelled cyclopamine. [d] See Table S2 for details. [e] All other saturated analogues **27** showed no Hedgehog pathway inhibition. n.d. = not determined.

structural relevance of the δ-lactone ring to Hh inhibition, further urethane analogues, for instance, derivatives that are based on alkyl carbamates instead of aryl carbamates or lack unsaturation and/or a hydroxymethyl substituent on the lactone ring, were synthesized (Scheme 3). To this end, palladium-mediated hydrogenation of lactone **15** provided the saturated α-substituted analogue **23** in 77% yield (major diastereomer shown). Reaction of the secondary alcohol of **23** with various isocyanates followed by desilylation yielded the desired withanolide analogues **24**. Withanolide analogues with completely unsubstituted and saturated δ-lactone rings were prepared by ring-closing metathesis of precursor **25**, followed by a stereoselective reduction of the ketone to deliver secondary alcohol **26**, which was converted into several carbamates **27** and **28** (Scheme 3; see the Supporting Information for details).

The osteogenesis assay revealed that the saturated urethane analogues that carry a hydroxymethyl group (compounds **24**) were weaker inhibitors (Table 1 and Table S2). Saturated compounds **27**, which lack the hydroxymethyl group, were mostly inactive. δ-Lactone **28**, which does not carry an additional substituent at the unsaturated double bond, was found to be cytotoxic presumably because of its higher electrophilicity. Replacement of the aryl carbamate by an alkyl carbamate (**21f–21l**) induced cytotoxicity and reduced the Hh inhibitory activity. The Smo binding potency of these molecules was therefore not evaluated (see Table S2).

Competition with cyclopamine in binding to Smo was further confirmed for the active Hh inhibitor **21a** by displacement of BODIPY–cyclopamine in HEK293T cells ectopically expressing Smo. Compound **21a** reduced the amount of Smo-bound BODIPY–cyclopamine as detected by means of microscopy and flow cytometry (Figure 2c,d; see also Figure S2).

In summary, a withanolide compound collection with the characteristic *trans*-hydrindane dehydro-δ-lactone scaffold was stereoselectively synthesized. The synthesis strategy includes the preparation and selective functionalization of three key intermediates. A biological investigation of the collection revealed novel and potent inhibitors of the Hedgehog signaling pathway, which bind to the Smoothened protein.

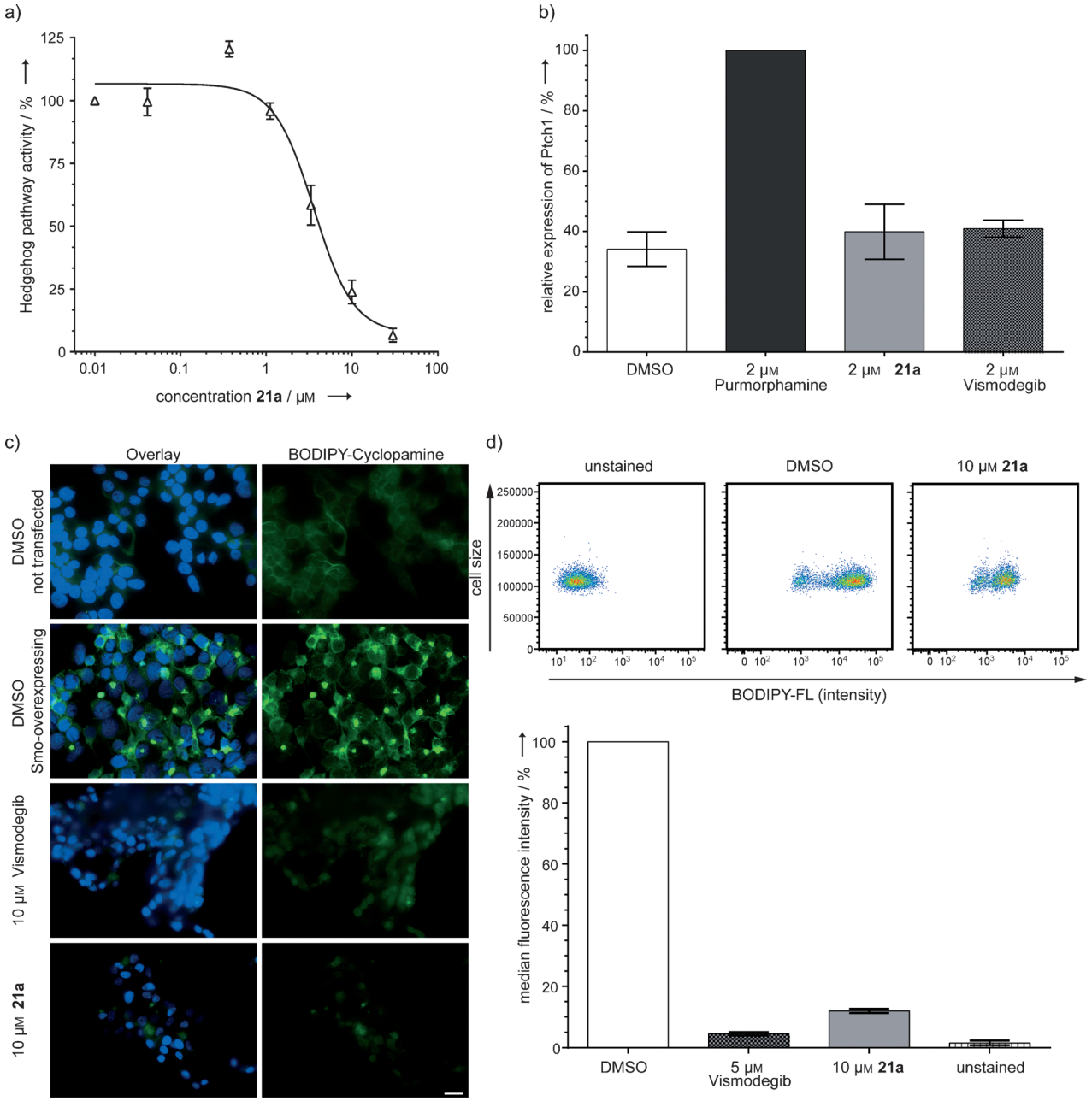
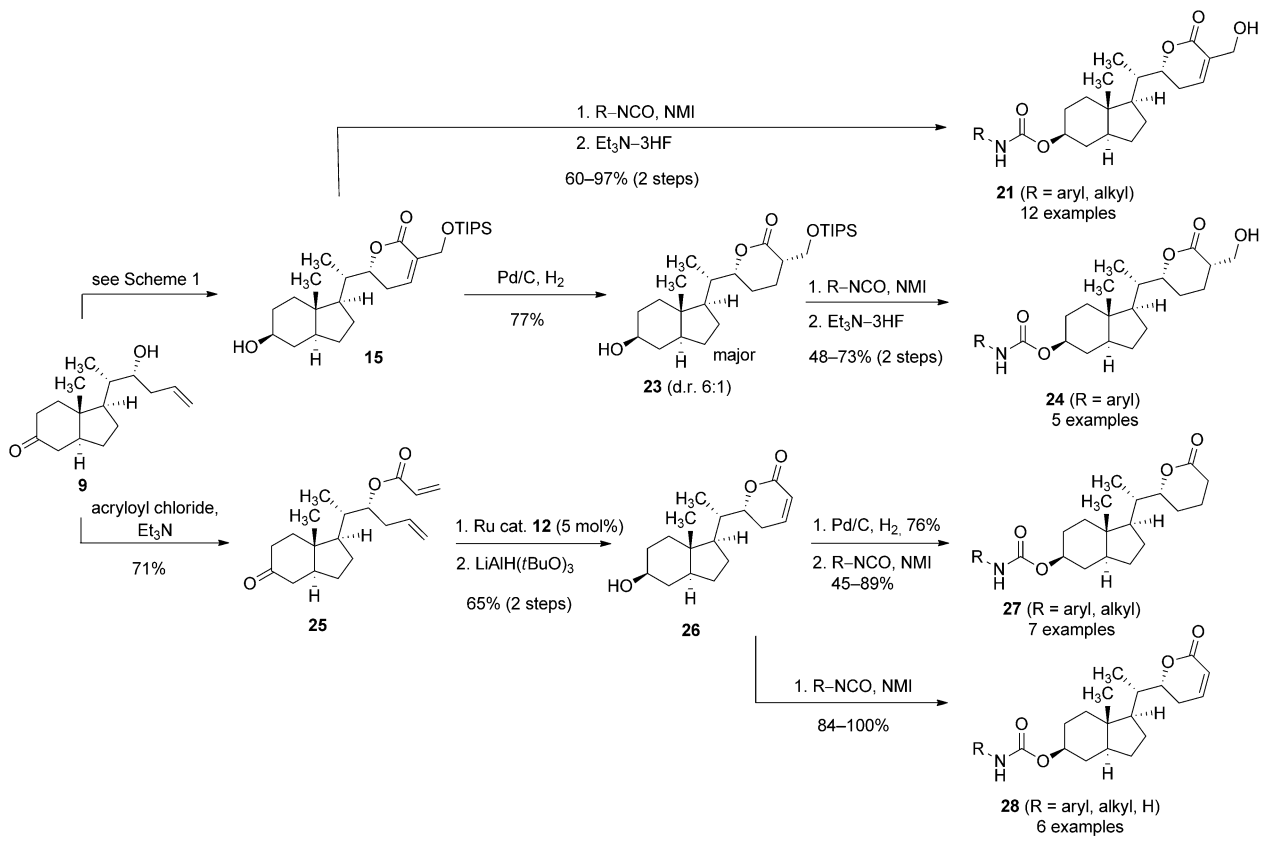


Figure 2. Compound **21a** inhibits Hedgehog signaling. a) Compound **21a** inhibits Gli-dependent reporter gene expression. SHH-LIGHT2 cells were treated with purmorphamine (4 μM) and different concentrations of **21a** or DMSO as a control for 48 hours. Firefly luciferase/Renilla luciferase ratios were determined. Values are expressed as the percentage of DMSO-treated cells. Nonlinear regression was performed using a four-parameter fit. Data are mean values ± standard deviation ($n = 3$). b) Treatment with compound **21a** leads to a decrease in the expression of the Hedgehog target gene Patched1 (Ptch1). NIH/3T3 cells were incubated with purmorphamine (2 μM) and different concentrations of **21a** or vismodegib or DMSO as a control for 48 hours. Upon cell lysis and cDNA preparation, quantitative PCR was carried out employing specific oligonucleotides for Ptch1 or Gapdh as a reference gene. Expression levels of Ptch1 were normalized to the levels of Gapdh and are given as the percentage of purmorphamine-activated cells (100%). Data are given as mean values ± standard deviation ($n = 3$). c, d) Compound **21a** interferes with the binding of BODIPY–cyclopamine to Smo. c) HEK293T cells were transfected with a Smo expression plasmid. Two days later, cells were fixed and treated with **21a** or vismodegib or DMSO as control in the presence of BODIPY–cyclopamine (5 nM) for four hours. Cells were then stained with DAPI (blue) to visualize the DNA. Scale bar: 20 μm. d) HEK293T cells were transfected with a Smo expression plasmid. Two days later, cells were treated with **21a** or DMSO as a control in the presence of BODIPY–cyclopamine (5 nM) for five hours. Cells were then subjected to flow cytometry analysis to detect Smo-bound BODIPY–cyclopamine. The graph shows the median BODIPY–cyclopamine fluorescence intensity of Smo-transfected cells upon treatment with the compounds. Data are mean values ± standard deviation ($n = 3$) and are presented as the percentage of DMSO-treated cells (100%).



Scheme 3. Synthesis of a collection of urethane-substituted *trans*-hydrindane dehydro-δ-lactone derivatives.

Keywords: biology-oriented synthesis · chemical biology · hedgehog pathway · natural products · total synthesis

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 5596–5602
Angew. Chem. **2015**, *127*, 5688–5694

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Received: January 6, 2015
Published online: March 3, 2015

