

Enhancement of Cyclopamine via Conjugation with Nonmetabolic Sugars

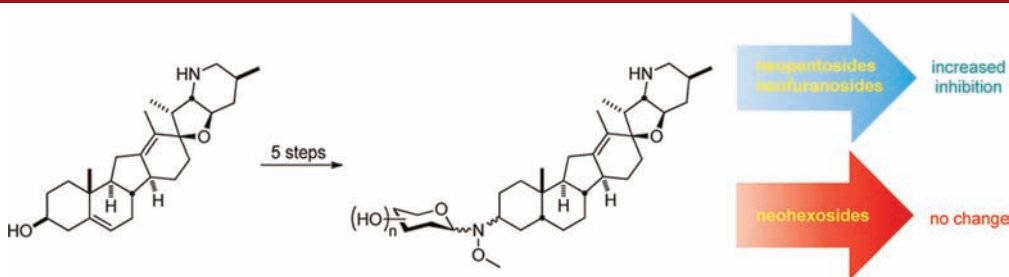
Randal D. Goff^{*,†} and Jon S. Thorson^{*,‡}

Department of Chemistry, University of West Florida, 11000 University Parkway, Pensacola, Florida 32514, United States, and Center for Pharmaceutical Research and Innovation, University of Kentucky College of Pharmacy, 789 South Limestone Street, Lexington, Kentucky 40536-0596, United States

rgoff@uwf.edu; jsthorson@uky.edu

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ABSTRACT



The *Veratrum* alkaloid cyclopamine, an inhibitor of cancer stem cell growth, was used as a representative scaffold to evaluate the inhibitory impact of glycosylation with a group of nonmetabolic saccharides, such as D-threose. In a five-step divergent process, a 32-member glycoside library was created and assayed to determine that glycosides of such sugars notably improved the GI₅₀ value of cyclopamine while metabolic sugars, such as D-glucose, did not.

Glycosylation is an important contributor to the biological activity of macromolecules (e.g., proteins and lipids)¹ and pharmaceutically active agents (e.g., natural products and synthetic drugs). In the latter case, sugars attached to small molecule-based drugs can dramatically influence the corresponding mechanism, pharmacodynamics, pharmacokinetics, and even patent life of the parental structure.² Given this precedent, the development of convenient strategies to synthesize glycoconjugate libraries are anticipated to facilitate the application of glycodiversification in drug lead identification and optimization.³ Neoglycorandomization, a divergent chemoselective

glycosylation method, is advantageous in this regard as it employs free reducing sugars and thereby avoids the need for subsequent postglycosylation modification/deprotection to produce the desired glycoconjugate.⁴ A survey of neoglycosylation studies to date—in the context of natural products,⁵ synthetic drugs,⁶ aglycon-linking strategies,⁷ and chemoselective handle variation⁶—surprisingly reveals a core set of nonmetabolic sugars (i.e., sugars not known to be involved in human metabolic processes) that

[†] University of West Florida.

[‡] University of Kentucky.

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consistently improve the latent anticancer activity of a range of target scaffolds. This core set includes both D- and L-entantiomers of the aldopentoses, D-glucurono-6,3-lactone, and D-threose. While the underlying mechanism of anticancer enhancement in some of the cases investigated to date remains unclear, this intriguing trend suggests a focused library biased toward inclusion of these core sugars may offer the greatest chance for anticancer improvement of a given target scaffold.

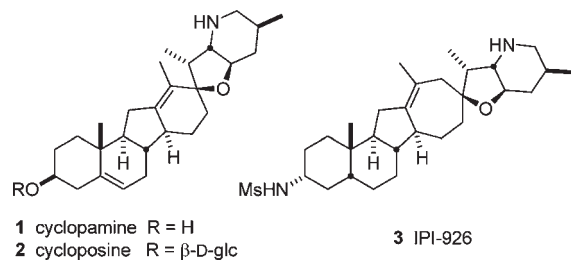


Figure 1. Structures of cyclopamine (**1**), cycloposine (**2**), and IPI-926 (**3**).

To test this hypothesis, we selected the *Veratrum* alkaloid cyclopamine (Figure 1, **1**) as a representative model. Identified in the 1960s as a teratogen capable of inducing cyclopia and holoprosencephaly in sheep embryos,⁸ cyclopamine disrupts natural embryonic development by binding to Smoothed, a key regulatory component of the Hedgehog (Hh) pathway,⁹ which is conserved in various organisms including humans.¹⁰ Because the Hh pathway is also responsible for proper division of adult stem cells, abnormal constitutive activation of Smoothed ultimately leads to an increase in the cancer stem cell population. Small molecule inhibitors such as **1** act to reverse this detrimental effect and thus function as anticancer agents without being directly cytotoxic.¹⁰ Tremblay et al. reported that small changes at the C3 position of D-ring-expanded

cyclopamine, including functional group attachment (e.g., sulfonamide, acetamide), lowered Hh inhibitory EC₅₀ values to nanomolar concentrations.¹¹ The most potent analog, IPI-926 (Figure 1, **3**),¹² is presently in six clinical trials for assessment in single- and combination-drug therapies against various types of cancer.¹³ As support for glycoconjugation, numerous veratramine, cevanine, and jervine (e.g., **1**) alkaloids from the common family of *Liliaceae* have also been isolated as β -D-glucosides,¹⁴ some of which invoked increased cAMP phosphodiesterase activity over their aglycon counterparts.¹⁵ One such compound, cycloposine (Figure 1, **2**), the 3-O- β -D-glucoside of cyclopamine, is known to impact embryonic development via Hh inhibition.^{8b,16} Glycosylation at the F-ring amine, via Huisgen 1,3-dipolar cycloaddition-mediated installation of anomeric azido sugars,¹⁷ or installation of hydrophobic moieties at the same site, also led to **1** improvements.^{9b,11a} Herein we report the synthesis of a suitable cyclopamine neoglycon in four steps and the subsequent creation of a 32-member library of cyclopamine neoglycosides. Evaluation of tumor cell growth inhibition revealed that the core group of nonmetabolic sugar derived glycosides induced a greater cancer cell growth inhibition than those deriving from typical metabolic sugars. Neoglycosylation also increased the solubility of cyclopamine. Consistent with prior observations, this study supports the idea that particular glycosides offer distinct advantages in glycoconjugation directed toward improving anticancer activity, the general mechanism of which warrants further investigation.

To prepare the cyclopamine neoglycon, oxidation at the C3 position was found to best occur under Oppenauer conditions as previously reported^{11a} due to product decomposition using TPAP/NMO and the potential for opening the spirofuran ring under Jones oxidation conditions (see Scheme 1). Upon oxidation, a 1,2-vinyl shift of the native double bond to C4 and C5 led to the newly

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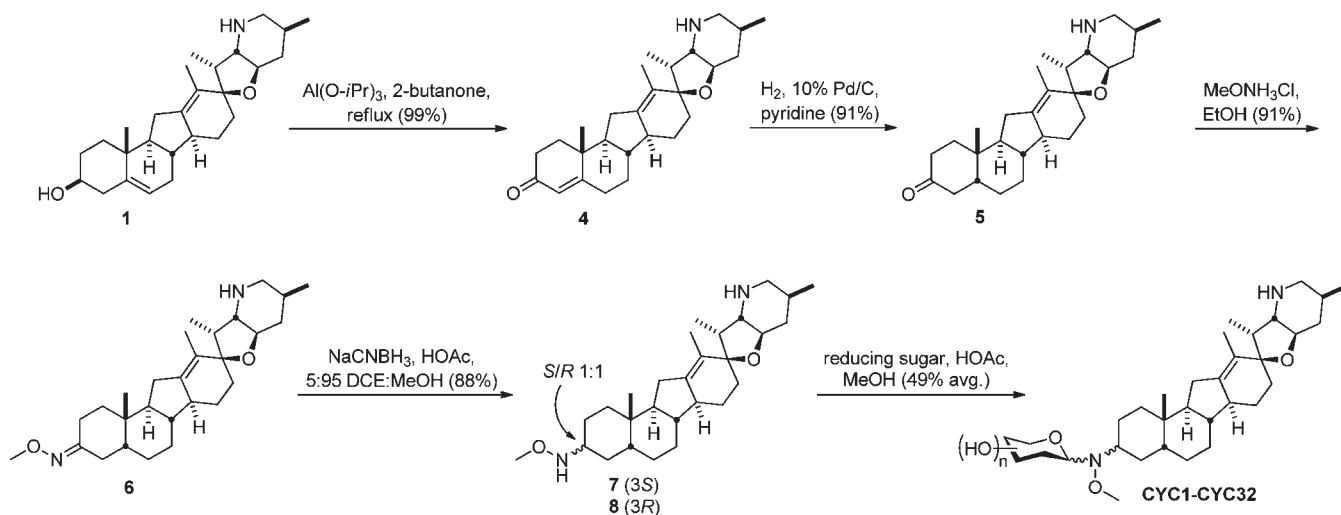
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Scheme 1. Synthesis of Cyclopamine Neoglycons (**7** and **8**) and Neoglycosides (**CYC1**–**CYC32**)



formed α,β -unsaturated ketone (**4**). Due to the lack of susceptibility of this conjugated system to reductive amination, the C4–C5 alkene was subsequently reduced by hydrogenolysis using a palladium–carbon catalyst. Rather than as a mixture of diastereomers, this reaction proceeded stereospecifically, providing the *cis*-decalone **5** as the only observable product. In support of this assignment, spectroscopic data of **5** were consistent with the similarly reduced D-ring-expanded analog described by Tremblay et al.^{11b} Methoxyimine **6** was subsequently generated by reacting **5** with methoxylamine HCl salt in the presence of a weak organic base, and the corresponding imine reduced using sodium cyanoborohydride with acetic acid in catalytic amounts. These reduction conditions, which were much milder than those with borane-amine complexes requiring HCl activation, were also found to open the spirofuran ring when not quenched properly prior to chromatographic purification. This was avoided by vigorously washing the reaction with an equal volume of saturated aqueous NaHCO₃ for at least 5 minutes. Unlike stereoselective C4–C5 alkene reduction, imine reduction led to a separable 1:1 mixture of C3 diastereomers (**7** and **8**). The stereochemical assignment at C3 for each diastereomer was based upon a comparison of chemical shifts of the C3 protons (3*S*: 3.25–3.18 ppm, multiplet; 3*R*: 2.81–2.73 ppm, multiplet) and carbons (3*S*: 55.23 ppm; 3*R*: 60.43 ppm), which were consistent with previously reported values.^{11b,18} Attempts to induce stereoselective reduction of the imine through alternate means (e.g., Na(AcO)₃BH; NaBH₄/CeCl₃·7H₂O; K-selectride) were unsuccessful. However, having access to both diastereomers also provided the means to assess the impact of the C3 stereocenter upon activity in the context of the corresponding neoglycosides. It is important to note that replacing EtOAc with CH₂Cl₂ as an extraction solvent during purification steps led to a dramatic increase

in the overall isolated yield of aglycons **7** and **8** over the four-step pathway from 23% to 72%.

With suitable neoglycons in hand, neoglycosylation was performed under mild conditions (i.e., MeOH/HOAc 8/1, 40 °C)^{6,7} to facilitate the chemoselective ligation in the presence of the spirofuran ring. To compare with the historically active set of glycosides (i.e., pentoses, D-threose, and D-glucurono-6,3-lactone) to alternative variants, carbohydrates that either had not previously induced increased cytotoxicity as neoglycosides or had not consistently done so, notably unmodified hexoses and deoxyhexoses, were also included within the library synthesis. A 32-member library (**CYC1**–**CYC32**) with 16 unique sugars was thus created (see Figure S1, Supporting Information (SI)) with an average isolated yield of 49%. Interestingly, when acetic acid was used in great excess (e.g., 25 equiv) of aglycon, the yields typically doubled over the same reaction time (4–28 h) without detriment to spirofuran opening. NMR revealed anomeric stereoselectivity to be dictated by the sugar employed as previously observed.^{5–7} Notably, the cyclopamine C3 configuration had no impact upon anomeric specificity, a phenomenon not previously observed in the context of other natural product-neoglycoside conjugates. In general, the neoglycoside anomeric configuration was biased toward a 1,2-*trans* relationship, with the exception of D-threosides **CYC1** and **CYC17**, D-lyxosides **CYC5** and **CYC21**, and L-lyxosides **CYC6** and **CYC22** (see Table S1, SI for characterization data).

To evaluate the impact of cyclopamine C3 neoglycosylation on biological activity, all 32 library members, along with aglycons **7** and **8** and parent compound **1**, were evaluated for growth inhibition using the representative lung cancer cell line NCI-H460.¹⁹ A preliminary two-dose growth inhibitory assay (10 and 100 μ M) revealed 5 of

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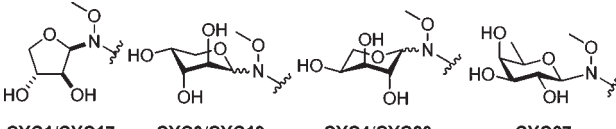
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the 32 cyclopamine neoglycosides to display $\geq 20\%$ inhibition at $10 \mu\text{M}$ (see Table S2, SI). Intriguingly, there was a disparity of activity between the two diastereomeric groups at this concentration that appeared to be independent of the cyclopamine C3 configuration. Specifically, with the exception of the similarly potent D-fucosides **CYC11** and **CYC27**, active compounds were identified from both the (3*S*)-**7** and (3*R*)-**8** aglycons for which existed notably less active diastereomeric counterparts. Based upon this preliminary comparison, seven neoglycosides were subsequently used for a full dose study guided by the following rationale: D-threosides **CYC1/CYC17** and D-arabinosides **CYC3/CYC19** to compare the impact of the C3 stereocenter in the context of the most active sugars; L-arabinosides **CYC4/CYC20** to compare to the corresponding enantiomers **CYC3/CYC19**; and D-fucoside (**CYC27**) as the most active hexose representative (see Table 1). As parental comparators, (3*S*)-aglycon **7**, (3*R*)-aglycon **8**, and **1** were also included.

The full dose study revealed all neoglycosides to be markedly improved over the parent **1**, having growth inhibition of 50% of the cellular population (i.e., GI_{50}) 3.5 to 12 times lower with D-arabinoside **CYC19** as the most active neoglycoside ($6.4 \mu\text{M}$). Consistent with our hypothesis, sugars previously found to consistently increase the anticancer activity of unrelated scaffolds^{5–7} led to the best cyclopamine improvements and little or no improvement was found via conjugation to representative metabolic sugars (e.g., D-Gal and D-Glc had no observable inhibition at $10 \mu\text{M}$). Only in the case of the most active sugar did the configuration at C3 effect a notable impact on GI_{50} values (**CYC3/CYC19**: 21 vs $6.4 \mu\text{M}$, respectively). Surprisingly, both neoaglycons **7** and **8** displayed improvements similar to that of the best neoglycoside **CYC19**. Deletion of the C5–C6 double bond likely did not have a deleterious effect consistent with previously reported 5,6-dihydro analogs of **1**.¹¹ While neoglycosides and neoaglycons were equipotent, glycosylation greatly improved solubility of the target scaffold. In DMSO, the solubility of neoglycosides **CYC1–32**, aglycons **7** and **8**, and **1** was ≥ 200 , < 50 , and < 4 mM, respectively. As cyclopamines modified at the A-ring with small molecular appendages (i.e., **3**) have been reported to have comparatively lower clearance and higher oral bioavailability,^{11b} the alterations in physical properties via neoglycosylation (as reflected by solubility differences) may offer added benefit in the context of cyclopamine-based therapeutic development.

In summary, this study highlights a rapid strategy for differential glycosylation of cyclopamine, a naturally

Table 1. GI_{50} Data of Cyclopamine Neoglycosides Against NCI-H460 Lung Cancer Cell Line



| entry | neoglycoside | C3 ^a | GI_{50} ^b |
|--------------|-----------------------|-----------------|------------------------|
| CYC1 | D-threoside | <i>S</i> | 8.5 ± 0.4 |
| CYC3 | D-arabinoside | <i>S</i> | 21 ± 1 |
| CYC4 | L-arabinoside | <i>S</i> | 17 ± 1 |
| 7 | (3 <i>S</i>)-aglycon | <i>S</i> | 5.5 ± 0.3 |
| CYC17 | D-threoside | <i>R</i> | 10.1 ± 0.9 |
| CYC19 | D-arabinoside | <i>R</i> | 6.4 ± 0.5 |
| CYC20 | L-arabinoside | <i>R</i> | 16 ± 1 |
| CYC27 | D-fucoside | <i>R</i> | 9.9 ± 0.4 |
| 8 | (3 <i>R</i>)-aglycon | <i>R</i> | 4.1 ± 0.1 |
| 1 | cyclopamine | <i>S</i> | 76 ± 5 |

^a Configuration at the aglycon C3 position. ^b GI_{50} values in μM .

occurring inhibitor of the Hh signaling pathway. Conjugation of cyclopamine with nonmetabolic sugars previously found to improve the potency of a range of structurally and mechanistically unrelated anticancer agents led to glycosides with greater overall improvements in both anticancer activity and solubility and suggest the improvements invoked by such sugars may in part be due to a general (e.g., transport) rather than target-specific mechanism. This work extends the trend of cyclopamine improvement via small-molecule conjugation at C3 to include neoglycosylation and may point to a benefit of similar C3 glycosylation in the context of the acid-stable expanded D-ring (e.g., **3**) cyclopamine series.

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Supporting Information Available. Experimental procedures, characterization data, structures, and growth inhibition data for **CYC1–CYC32** and aglycons **7** and **8**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare the following competing financial interest(s): J.S.T. is a co-founder of Centrose (Madison, WI).