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Brief Article

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The Identification of Perillyl Alcohol Glycosides with Improved Antiproliferative Activity

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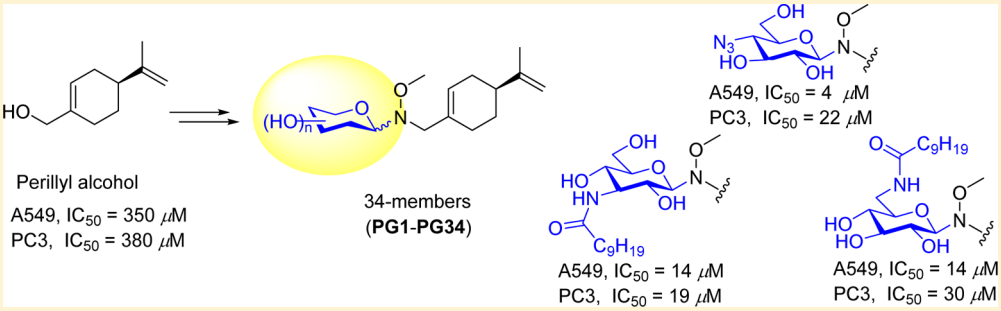
Supporting Information



ABSTRACT: A facile route to perillyl alcohol (POH) differential glycosylation and the corresponding synthesis of a set of 34 POH glycosides is reported. Subsequent in vitro studies revealed a sugar dependent antiproliferative activity and the inhibition of S6 ribosomal protein phosphorylation as a putative mechanism of representative POH glycosides. The most active glycoside from this cumulative study (4′-azido-D-glucoside, **PG9**) represents one of the most cytotoxic POH analogues reported to date.

INTRODUCTION

(*S*)-Perillyl alcohol (POH, also known as *p*-metha-1,8-diene-7-ol or 4-isopropenyl cyclohexene carbinol) is a monoterpene produced by a number of plants, including cherries, lavendin, mints, and celery seeds via oxidative modification of D-limonene.[1,2] Interest in POH as a potential anticancer agent stems from its ability to cause G1 cancer cell cycle arrest via the putative inhibition of post-translational modification of signal transduction proteins involved in the Ras/MAPK pathway.[3] While the fundamental mechanism(s) and/or target(s) of POH remain unclear, POH has been implicated in a range of functions including inhibition of small G protein isoprenylation and induction of proto-oncogenes,[4] inhibition of Na/K-ATPase,[5] disruption of hTERT−mTOR−RAPTOR protein complex,[6] suppression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase synthesis in mammalian cells,[2,7] as well as inhibition of 4E-BP1(Ser65) phosphorylation and cap-dependent translation.[8] POH was initially evaluated in phase I and phase II clinical trials for the treatment of a range of cancers (breast, colon, ovarian, and prostrate).[9−16] While these studies revealed POH to be well tolerated at high doses, tumor responses were low. However, more recent clinical studies focused upon intranasal delivery of POH to treat recurrent malignant glioblastoma led to notable tumor regression with high doses of POH.[17−19] This promising precedent suggests that POH analogues with improved potency and/or drug properties may serve to reinvigorate the clinical utility of this unique plant metabolite and also potentially offer new probes for further mechanistic interrogation.

The glycosylation of small-molecule-based or natural product-based leads/drugs can often dramatically influence pharmacological properties and ADMET.[20] In the context of POH, a small set of naturally occurring POH glycosides (Figure 1; **1a**, **2a**, **9a**, **10a**) isolated from *Perilla frutescens* leaves was first reported as aldose reductase inhibitors.[21−24] A few additional POH glycosides were generated via conventional or enzymatic synthesis,[21,25−27] (Figure 1; **3a**−**8a**, **11a**) and, cumulatively, these studies revealed certain POH glucosides (Figure 1; **1a**, **3a**) as equipotent to POH against select cancer cell lines in vitro. Yet, studies designed to systematically assess and/or exploit the impact of POH glycosylation are lacking. Neo-glycorandomization, a divergent chemoselective glycosylation method, is advantageous in this regard as it offers a rapid strategy for differential glycosylation of a selected target scaffold.[28] Herein we report the synthesis and in vitro anticancer activity of a set of 34 distinct POH neoglycosides, several of which displayed improved in vitro anticancer activity over the parent natural product. The best among these (4′-azido-D-glucoside **PG9**) displayed a striking enhancement in

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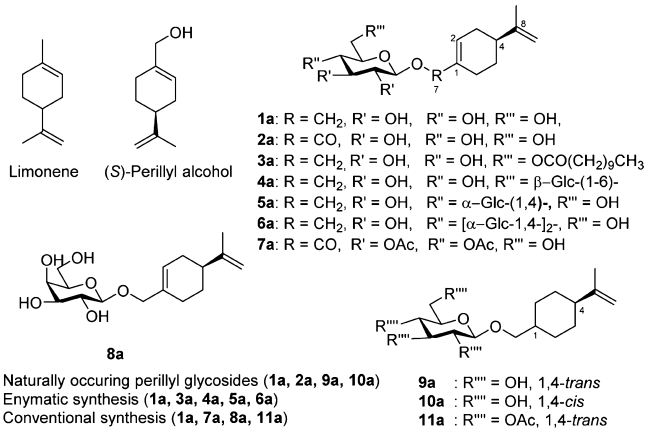


Figure 1. Limonene, perillyl alcohol (POH), and previously reported POH glycosides.

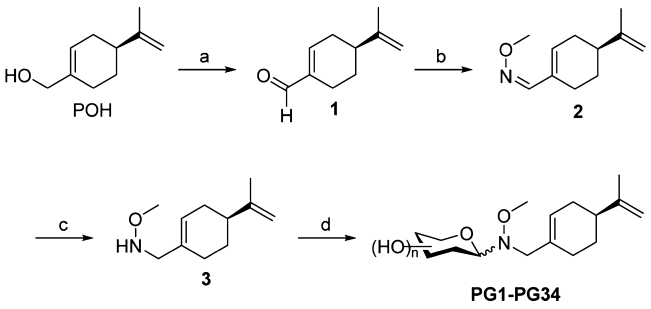
potency (>85-fold against A549 nonsmall cell lung; 15-fold against PC3 prostate) over POH and represents the most active perillyl glycoside reported to date. In contrast to previous reports,[8] the subsequent study of the influence of POH and representative improved POH glycosides upon 4E-BP1 phosphorylation in A549 cells suggests the antiproliferative effects of the improved POH glycosides may be associated with the inhibition of cap-dependent translation by targeted inhibition of phosphorylation of S6 ribosomal protein rather than 4E-BP1. This study also highlights, for the first time, the compatibility of neoglycosides with Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition.

RESULTS AND DISCUSSION

Perillyl neoaglycon **3** was synthesized in three simple steps (Scheme 1). Specifically, POH was oxidized to (*S*)-perillaldehyde (**1**) using *o*-iodoxybenzoic acid (IBX) and acetic acid.[29] Subsequent reductive amination proceeded via formation of oxime **2** with methoxyamine hydrochloride in the presence of triethylamine, followed by reduction with $NaCNBH_3$ in the presence of acetic acid to provide the requisite neoaglycon **3**.[30] Optimization of **3** neoglycosylation with D-glucose using a range of general neoglycosylation conditions[31−33] revealed MeOH:HOAc (5:1) as most effective, providing the corresponding D-glucoside **PG1** in 69% isolated yield and, consistent with prior studies,[34] the β-anomer exclusively. The scope of sugars selected for this study included representative pentoses (D/L-arabinose, **PG3**/**PG28**; L-ribose, **PG17**; D-xylose, **PG22**), hexoses (D/L-glucose, **PG1**/**PG6**; 3-*O*-methyl-D-glucose, **PG4**; L-rhamnose, **PG32**), azidosugars (6-deoxy-6-azido-D-glucose, **PG2**; 3-deoxy-3-azido-D-glucose, **PG8**; 4-deoxy-4-azido-D-glucose, **PG9**; 2-deoxy-2-azido-D-glucose, **PG13**; 4,6-deoxy-4,6-diazido-D-glucose, **PG20**, 4-deoxy-4-azido-L-glucose, **PG24**; 4-deoxy-4-azido-D-xylose, **PG34**), fluorosugars (3-deoxy-3-fluoro-D-glucose, **PG18**; 4-deoxy-4-fluoro-D-glucose, **PG21**; 2-deoxy-2-fluoro-D-glucose, **PG29**; 2-deoxy-2-fluoro-D-mannose, **PG30**) *N*-acyl sugars (*N*-acetyl-D-galactosamine, **PG5**; *N*-acetylmuramic acid, **PG12**; streptozocin, **PG16**; 3-*N*-decanoyl-D-glucosamine, **PG23**; 6-*N*-decanoyl-D-glucos amine, **PG26**; 3-*N*-allyloxylcarbonyl-D-glucosamine, **PG27**; 2-*N*-allyloxylcarbonyl-D-glucosamine, **PG31**; 6-*N*-allyloxylcarbonyl-D-glucosamine, **PG33**), an acid-bearing sugar (D-glucuronic acid, **PG25**), and a dissacharide (D-cellobiose, **PG14**) with a bias toward glucosides and acyl glucosides based upon the previously reported active glycosides (**1a** and **3a**). The inclusion of azidosugars served as a starting point for subsequent divergence via chemoselective modification [via Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition][35,36] or selective reduction to afford the corresponding aminosugar conjugates (Scheme 2).[30,32,37−39] A library of 34 distinct neoglycosides (**PG1**−**PG34**) were synthesized (Figure S1 and Table S1 in Supporting Information) in good to excellent yields (23−82%), with the β-anomer as predominate product in most cases, consistent with previous studies.[40−49] For aminosugar conjugates, azidosugar glycosides (**PG2**, **PG8**, **PG9**, **PG13**) were readily reduced to their corresponding aminosugar counterparts (**PG7**, **PG10**, **PG11**, **PG19**) in the presence of PMe_3 (1.0 M in THF), with yields ranging from 29 to 46%. To assess the compatibility of neoglycosides with Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition and the potential impact of sugar triazole substitution upon POH bioactivity, the 6′-azido perillyl glucoside was also converted to the corresponding 6′-triazole perillyl glucoside **PG15** via Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition in 82% yield (Scheme 2).

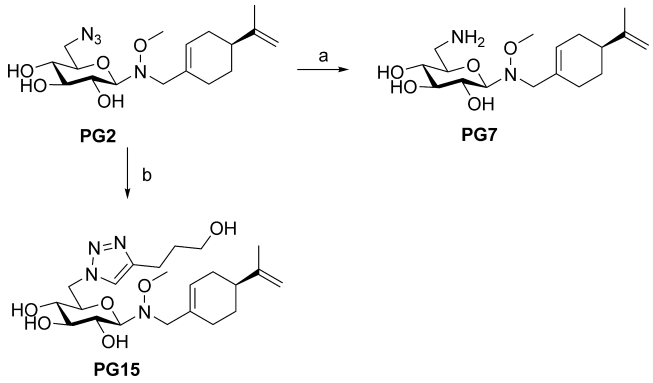
The anticancer properties of POH, **1**, **2**, **3**, and **PG1**−**PG34** against two human cancer cell lines (nonsmall-cell lung A549 and prostate PC3) was first assessed via percent inhibition of cell viability at a single dose of 250 μM (Figure 3). Compounds which displayed >40% inhibition in one or both cell lines

Scheme 1. Synthesis of Perillylneoaglycon (3) and Neoglycosides (PG1−PG34)[a]



[a]Reagents and conditions: (a) IBX, HOAc, acetonitrile (88%); (b) $MeONH_3Cl$, Et_3N, EtOH (84%); (c) $NaCNBH_3$, HOAc, MeOH (50%); (d) reducing sugar, MeOH:HOAc (5:1), 40 °C (23%−77%).

Scheme 2. Divergent Transformations of Azidosugar-Bearing Neoglycoside PG2[a]



[a]Reagent and conditions: (a) PMe_3 in THF, overnight, (46%); (b) 4-pentyn-1-ol, CuI, Et_3N, ACN, overnight, (82%).

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(oxime **2**, **PG2**, **PG9**, **PG13**, **PG16**, **PG20**, **PG22**, **PG23**, **PG24**, **PG26**, **PG31**, **PG33**, **PG34**) from the single dose analysis were subsequently subjected to a full dose study for IC_{50} determination along with the parental POH (Figure 2, 4).

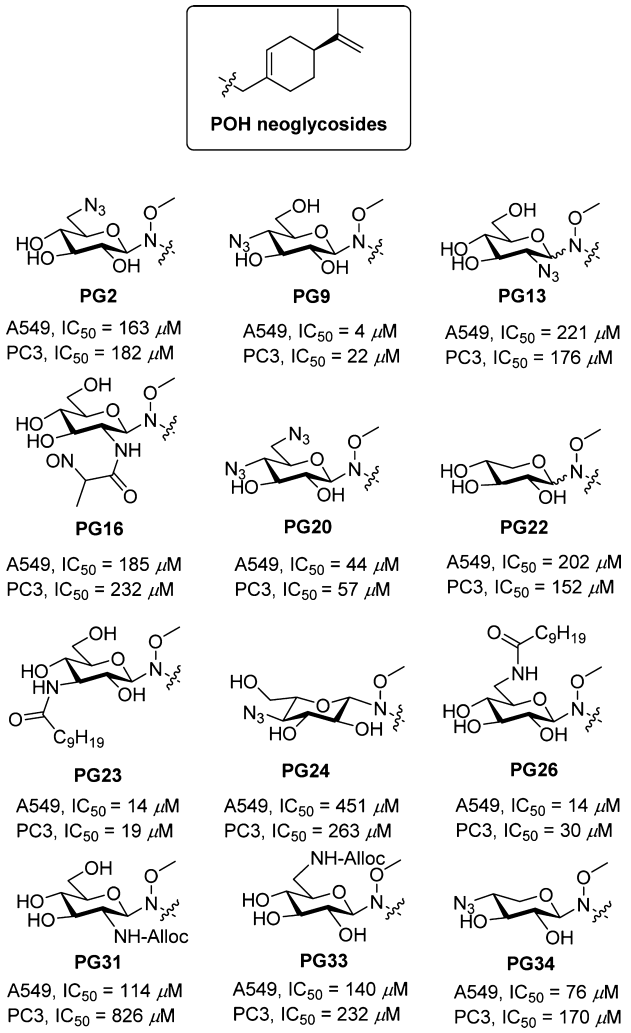

Figure 2. Structures of the most antiproliferative POH neoglycosides against A549 and PC3 cell lines.

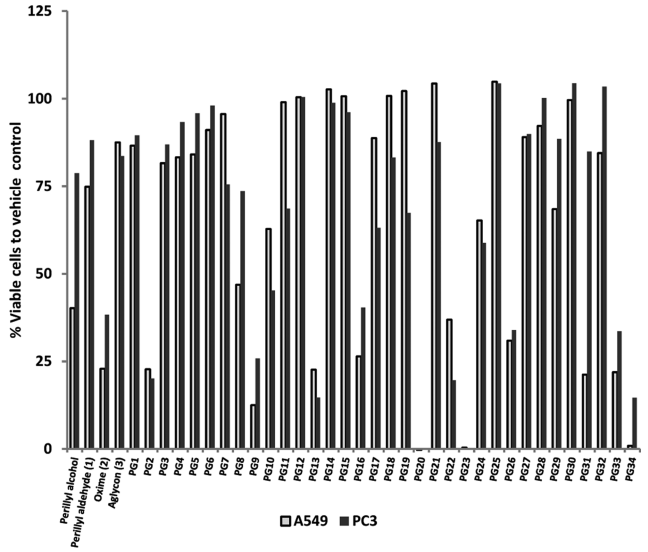

Figure 3. (a) Single dose (250 μM) comparisons of POH neoglycosides and POH against A549 and PC3 cell lines.

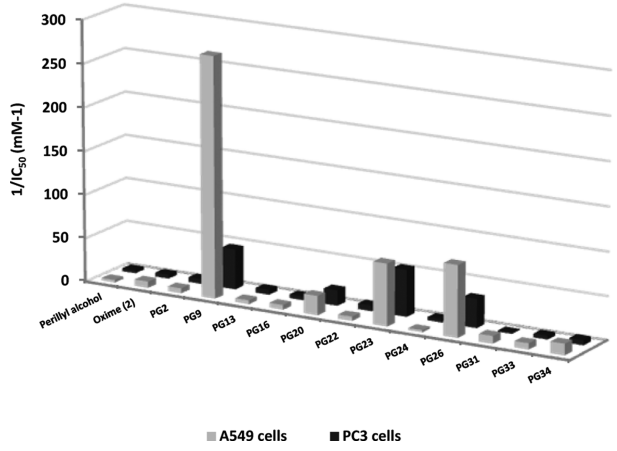

Figure 4. Reciprocal IC_{50} values for POH (IC_{50} 350 μM, A549; 380 μM, PC3) and the most active POH neoglycosides (the most active of which is **PG9**, with an IC_{50} 4 μM, A549; 22 μM, PC3). IC_{50} values and ± SD are summarized in Supporting Information, Table S2 and represent a triplicate of dose−response experiments conducted over nine concentrations at 2-fold dilution.

This broad analysis revealed the following key observational trends. First, substitutions of key sugar hydroxyls with an azide present the greatest improvements. For example, while D-glucoside **PG1** was relatively inactive (>500 μM in both cell lines), C6′ (**PG2**: 163 μM, A549; 182 μM, PC3), C4′ (**PG9**: 4 μM, A549; 22 μM, PC3), or C2′ (**PG13**: 221 μM, A549; 176 μM, PC3) azido substitution afforded moderate to dramatic improvements in potency over the parent natural product (POH: 350 μM, A549; 380 μM, PC3). From this series, the C4′-azidosugar variant **PG9** stands out as the best analogue, with >15-fold improved potency against PC3 and >85-fold increased potency against A549 in vitro, highlighting regiospecificity as an important contributor. Second, the benefits from sugar azido substitution are not additive, as evidenced by a comparison of **PG2** (C6′-azido) and **PG9** (C4′-azido) to the C4′,C6′-diazido analogue **PG20**. Third, while the facile synthesis of the C6′-triazole-substituted analogue **PG15** highlights the compatibility of neoglycosides with Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition chemistry, C6′-triazole substitution is detrimental to activity based upon the comparative activities of **PG2** (C6′-azido) and **PG15**. Fourth, sugar *N*-acylation also generally improved activity as both the 3′-*N*-decanoyl and 6′-*N*-decanoyl aminoglucosides (**PG23** and **PG26**, respectively) displayed relatively similar improvements in potency over POH (12−20-fold, PC3; 20−25-fold, A549) with shorter C2′, C3′ and C6′ *N*-acyl substitutions (e.g., **PG16**, **PG27**, **PG31**, **PG33**) generally being less favorable. Fifth, while the D-glucoside **PG1** displayed activity similar to the parent POH (in a manner reminiscent to the prior work with **1a**), removal of the C6′-CH_2OH (**PG22**) in this context led to slight improvements and, consistent with **PG9**, the addition of a 4′-azido-substitution (**PG34**: 76 μM, A549; 170 μM, PC3) led to further improvements in potency. Finally, on the basis of the comparative activities of **PG9** and **PG24**, the D-enantiomer offered the greatest enhancement.

While a number of putative anticancer mechanisms for POH have been put forth, one reported effect centers around cap-

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dependent translation in tumor cell lines. Specifically, Peffley and co-workers reported POH at 400 μM to suppress 4E-BP1 (Ser65/Thr37) phosphorylation and to disrupt interactions between critical components of the capped mRNA-binding complex (eIF4E and eIF4G).[8] Thus, the effect of POH and representative glycosides **PG20** and **PG23** on 4E-BP1 phosphorylation was assessed using the cell line in which the most dramatic antiproliferative improvements were observed (A549). As illustrated in Figure 5, in A549 cells POH had no

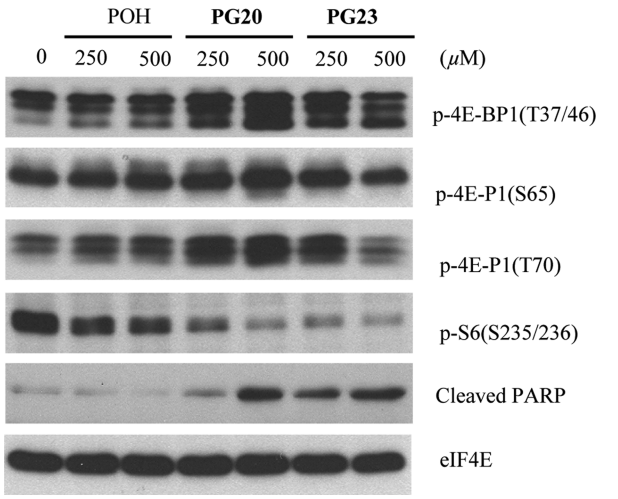

Figure 5. Western blot representing the effects of 250 and 500 μM perillyl alcohol (POH), **PG20**, and **PG23** treatments of A549 on phosphorylation of 4E-BP1 at 6 h.

effect upon the phosphorylation of 4E-BP1 at Thr37/46, Ser65, or Thr70, but 4E-BP1 phosphorylation was induced by **PG20** and **PG23**. Unlike POH, **PG20** and **PG23** also profoundly inhibited phosphorylation of S6 ribosomal protein, another important regulator of cap-dependent translation.[50] Furthermore, **PG20** and **PG23** substantially increased levels of cleaved PARP, an indicator of apoptosis, consistent with their increased antiproliferative activities. Cumulatively, these data suggest that the cytotoxicity of **PG20** and **PG23** toward A549 cells may be associated with the inhibition of phosphorylation of S6 ribosomal protein rather than 4E-BP1.

CONCLUSION

This study highlights a facile four-step process for POH glycodiversification and the discovery of a series of new POH glycosides that display improvements in potency approaching 2 orders of magnitude. For comparison, a survey of the previously reported activities of POH analogues (including glycosides, prodrug conjugates, and additional functionalized variations)[21−27,51−54] revealed C7-*N*,*N*-disubstituted amino derivatives as among the most potent to date, the best of which displayed an IC_{50} > 50 μM against A549. Thus, the activity of the POH 4′-azido-D-glucoside (**PG9**) reported herein stands out among most, if not all, POH derivatives reported to date. Cumulatively, the SAR presented suggests lipophilicity (azido[55] or *N*-acyl) to be of greatest benefit. While the fundamental antiproliferative mechanism of POH and POH congeners remains unclear, the current study also supports the contention that the newly reported POH glycosides may derive, at least in part, from the inhibition of phosphorylation of S6 ribosomal protein rather than 4E-BP1 as previously put forth.[8] Additional studies to specifically identify the target and assess the in vivo efficacy of these newly discovered POH glycosides are underway.

EXPERIMENTAL SECTION

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Sugar donors 2-deoxy-2-azido-D-glucose and 6-deoxy-6-azido-D-glucose were purchased from Carbosynth (Berkshire, UK). Solvents were of ACS grade and purchased from Pharmco-AAPER (Brookfield, CT). TLC silica gel plates (60 F254) were purchased from EMD Chemicals Inc. (Gibbstowm, NJ). NMR spectra were obtained on either a Varian Unity Inova 400 or 500 MHz instrument (Palo Alto, CA) at ambient temperature using 99.8% $CDCl_3$ and 99.8% CD_3OD (Cambridge Isotope Laboratories, MA, USA). ^1H and ^{13}C chemical shifts were referenced to internal solvent resonances and reported in parts per million (ppm), with coupling constants *J* given in Hz. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), dt (doublet of triplet), m (multiplet), and br (broad). HR-ESI-MS spectra were recorded on AB SCIEX Triple TOF 5600 system (AB Sciex, Framingham, MA, USA). Flash column chromatography was performed with RediSep *Rf* Gold columns from Teledyne Isco (Lincoln, NE) on a Biotage Isolera 4 (Biotage, Charlotte, NC, USA). Library member purity was assessed by ^1H NMR. The purity of starting material, (neoaglycon **3**) and all corresponding neoglycosides was determined to be ≥95% unless specified otherwise (Supporting Information, Table S1).

[(4*S*)-4-(Prop-1-en-2-yl)cyclohex-1-en-1-yl]methanol (1). A suspension of POH (1 g, 6.6 mmol), IBX (45 wt %, 3.7 g, 2.0 mmol), and acetic acid (0.75 mL, 2.0 mmol) in acetonitrile (5 mL) was stirred vigorously at room temperature overnight. After completion of the reaction based upon TLC, sodium bicarbonate (200 mg) was added. The resulting mixture was filtered and the solvent was removed in vacuo to afford the crude product, which was purified by normal-phase flash chromatography using *n*-hexane/ethyl acetate as eluent (0.9 g, 88% yield). ^1H NMR (500 MHz, $CDCl_3$) δ 9.41 (s, 1H), 6.81−6.79 (m, 1H), 4.73 (d, 2H), 2.49−2.39 (m, 2H), 2.25−2.17 (m, 2H), 2.13−2.06 (m, 1H), 1.90−1.86 (m, 1H), 1.74 (s, 3H), 1.45−1.39 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.0, 150.7, 148.4, 141.4, 109.6, 40.8, 31.8, 26.5, 21.7, 20.8. HRMS-ESI (*m*/*z*): calcd for $C_{11}H_{18}NO$ (MH^+) 180.1383, found 180.1385.

Methoxy({[(4*S*)-4-(prop-1-en-2-yl)cyclohex-1-en-1-yl]methylidene})amine (2). Perillyl aldehyde **1** (2 g, 13.3 mmol) was dissolved in absolute EtOH (40 mL). Et_3N (2.4 mL, 17.3 mmol) and methoxylamine hydrochloride (1.4 g, 17.3 mmol) were added, and the reaction was stirred overnight at room temperature. The solvent was removed in vacuo, and the crude product was purified by normal-phase flash chromatography using *n*-hexane/ethyl acetate as eluent (2.0 g, 84% yield). ^1H NMR (500 MHz, $CDCl_3$) δ 7.59 (s, 1H), 5.93 (t, 1H), 4.70 (d, 2H), 3.80 (s, 3H), 2.43 (dd, 1H), 2.29−2.11 (m, 3H), 2.09−2.01 (m, 1H), 1.86−1.80 (m, 1H), 1.70 (s, 3H), 1.48−1.38 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.5, 149.1, 134.8, 132.7, 109.1, 61.6, 41.0, 31.4, 26.9, 24.0, 20.8. HRMS-ESI (*m*/*z*): calcd for $C_{11}H_{18}NO$ (MH^+) 180.1383, found 180.1385.

Methoxy({[(4*S*)-4-(prop-1-en-2-yl)cyclohex-1-en-1-yl]methyl})amine (3). Oxime **2** (2.0 g, 11.2 mmol) was dissolved in MeOH (50 mL) under argon followed by the addition of $NaCNBH_3$ (7.0 g, 111.6 mmol) and HOAc (3.2 mL, 55.8 mmol). The reaction was stirred at room temperature for 48 h, subsequently quenched with saturated aqueous $NaHCO_3$ (45 mL), and MeOH was removed in vacuo. The aqueous layer was extracted with $CHCl_3$ (40 mL × 3). The recovered organics were washed with water and brine, dried over Na_2SO_4, and subsequently filtered and concentrated in vacuo to provide crude product which was purified by normal-phase flash chromatography using *n*-hexane/ethyl acetate as eluent (1.0 g, 50% yield). ^1H NMR (500 MHz, $CDCl_3$) δ 5.61 (s, 1H), 5.52 (s, 1H), 4.67 (s, 2H), 3.48 (s, 3H), 3.37 (s, 2H), 2.19−2.02 (m, 4H), 1.98−1.85 (m, 1H), 1.84−1.74 (m, 1H), 1.69 (s, 3H), 1.48−1.38 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 149.9, 134.0, 124.4, 108.7, 61.8, 58.1, 41.1, 30.7,

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27.7, 27.7, 20.9. HRMS-ESI (m/z): calcd for $C_{11}H_{20}NO$ (MH^+) 182.1539, found 180.1542.

General Neoglycosylation Procedure (PG1−PG6, PG8, PG9, PG12−PG14, PG16−PG18, PG20−PG34). Neoaglycon (**3**) was dissolved in MeOH/AcOH (5:1) in 1 dram vials along with stir fleas at a final concentration of 300−350 mM. Reducing sugar (2 equiv) was added and the vial capped, and the reaction was stirred at 40 °C for 24−48 h. Reaction progress was monitored by TLC. Upon completion, solvent was removed in vacuo to afford the crude product, which was purified by normal-phase flash chromatography using $CHCl_3$/MeOH as eluent. The perillyl neoglycosides were obtained with an isolated yield ranging from 23% to 77% from perillyl neoaglycon **3** (Supporting Information, Table S1). Anomeric ratios were obtained by comparison of anomeric proton integration (Supporting Information, Table S1).

General Procedure for Azide Reduction (PG7, PG10, PG11, PG19). To a 100 mM solution of azidosugar glycoside in THF was added PMe_3 in THF (1.2 equiv), and the reaction was stirred at 50 °C for 1 h. After removal of the solvent in vacuo, the crude residue was purified by normal-phase column chromatography using $CHCl_3$/MeOH as eluent to afford the desired aminosugar glycoside. The aminosugar-appended perillyl neoglycosides were obtained with an isolated yield yield ranging from 29% to 46% (Supporting Information, Table S1).

Cancer Cell Line Cytotoxicity Assay. Human cancer cell line (lung adenocarcinoma A549 and prostate cancer PC3) viability was determined using the alamar blue (or resazurine reduction) assay as previously described.[33,56−58] Briefly, cells were seeded in F-12K medium (Kaighn's Modification of Ham's F-12 medium, Invitrogen), supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/mL penicillin, and 100 μg/mL streptomycin onto flat-bottomed 96-well tissue culture plates (Corning, NY, USA) at a density of 3 × 103 cells/well and allowed to adhere overnight. After removing the medium, 100 μL of fresh medium containing nine 2- or 3-fold dilutions (500 to 75 nM) of each compound were added. All assays were conducted in the presence of both negative (0.5% DMSO vehicle control) and positive (1.5 mM H_2O_2) controls. The plates were incubated for 48 h under standard conditions, 5% CO_2 and 37 °C, in a humidity control incubator. At the end of incubation period, 10 μL of resazurin (0.25 mg/mL in water) was added to each well. Plates were incubated for an additional 3 h at standard culture conditions and shaken for 5−10 s, and fluorescence of resarufin (λ_{ex} = 600 nm, λ_{em} = 590 nm) was recorded as a basis for IC_{50} determination using a nonlinear interpolation of dose-dependent curves using Prizm. Data presented (Figure 4 and Supporting Information, Table S2) represent mean values (±SD) of triplicate determinations from three independent experiments.

Immunoblot Analysis. A549 cells were treated with 250 and 500 μM of perillyl alcohol (POH), **PG20**, and **PG23** for 6 h. Cells were harvested, lysed, and the corresponding extracts subjected to SDS-PAGE, and analyzed by Western analysis as previously described.[59] Each SDS-PAGE sample contained equal amounts of total protein. Commercial primary antibodies for p-4E-BP1(T37/46), p-4E-BP1-(S65), p-4E-BP1(T70), p-S6 (S235/236), eIF4E, and cleaved PARP (Cell Signaling Technology, Danvers, MA) were employed, and secondary antibodies were detected using chemiluminescence (GE Healthcare Bio-Sciences, Pittsburgh, PA).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic procedure of sugars, characterization data of synthesized compounds, and activity results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): The authors report competing interests. JST is a co-founder of Centrose (Madison, WI).

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■ ABBREVIATIONS USED

POH, (*S*)-perillyl alcohol; TLC, thin layer chromatography; IC_{50}, concentration of agent that inhibits cell viability by 50%

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