Published in final edited form as:

J Med Chem. 2010 November 25; 53(22): 8129–8139. doi:10.1021/jm101024j.

An Assessment of Chemoselective Neoglycosylation Methods Using Chlorambucil as a Model

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Abstract

To systematically assess the impact of glycosylation and the corresponding chemoselective linker upon the anticancer activity/selectivity of the drug chlorambucil, herein we report the synthesis and anticancer activities of a 63-member library of chlorambucil-based neoglycosides. A comparison of *N*-alkoxyamine-, *N*-acyl hydrazine- and *N*-hydroxyamine-based chemoselective glycosylation of chlorambucil revealed sugar- and linker-dependent partitioning among open and closed-ring neoglycosides and corresponding sugar-dependent variant biological activity. Cumulatively, this study represents the first neoglycorandomization of a synthetic drug and expands our understanding of the impact of sugar structure upon product distribution/equilibria in the context of *N*-alkoxyamino-, *N*-hydroxyamino- and *N*-acyl hydrazine-based chemoselective glycosylation. This study also revealed several analogs with increased in vitro anticancer activity, most notably *D*-threoside **60** (NSC 748747), which displayed much broader tumor specificity and notably increased potency over the parent drug.

Introduction

The sugars attached to pharmaceutically important natural products dictate the pharmacokinetics and/or pharmacodynamics of the selected agent.^{1–3} Yet, studies designed to systematically understand and/or exploit the attachment of carbohydrates in drug discovery remain limited by the availability of practical synthetic and/or biosynthetic tools.^{4–8} Neoglycosylation takes advantage of a chemoselective reaction between free reducing sugars and *N*-methoxyamino-substituted acceptors.^{9–19} This reaction has enabled the process of 'neoglycorandomization' wherein alkoxyamine-appended natural product-based drugs are differentially glycosylated with a wide array of natural and unnatural reducing sugars.^{4–8,20–24} Neoglycorandomization has led to the discovery of cardenolide neoglycosides with enhanced in vitro and in vivo anticancer activity and lower in vivo toxicity,^{20,23} colchicine neoglycosides with a novel anticancer mechanism and lower in vivo toxicity,²¹ vancomycin neoglycosides which displayed improved in vitro potency against clinical isolates of vancomycin-resistant *Enterococci*,²² and betulinic acid neoglycosides improved for either in vitro anticancer or antiviral potency.²⁴ While these examples clearly highlight the potential impact of differential glycosylation in drug lead discovery, this work has not addressed the impact of the chemoselective glycosylation 'handles' employed.

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Supporting Information Available Experimental procedures and characterization data for aglycons **9**, **65**, and **71**; characterization, figures, and growth inhibition data for the neoglycoside library; NCI60 anticancer screen data for **60**; and NMR spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

To assess the impact of alternative chemoselective glycosylation methods in the context of neoglycorandomization, we selected the synthetic anticancer agent chlorambucil (Figure 1, **1**) as a model. First synthesized over five decades ago,²⁵ chlorambucil remains a current treatment for chronic lymphocytic leukemia (CLL)^{26–30} and has served as the basis for newer generation analogs such as the recently approved bendamustine (**3**).³¹ A nitrogen mustard, **1** leads to guanine alkylation and DNA cross-linking, and ultimately prohibits DNA replication and transcription.^{32–33} The primary cellular uptake mechanism of **1** is passive diffusion³⁴ and like many cytotoxics, the lack of nitrogen mustard tumor-specificity contributes to serious side effects.^{35–36} Thus, improvements have focused upon *i*) the development of tumor-activated prodrugs – exemplified by the hypoxia-activated *N*-oxide PX-478 (Figure 1, **2**) currently in phase I;³⁷ or *ii*) modifications to engage tumor-specific transport – exemplified by the β -D-glucosyl analog of ifosfamide (glufosfamide, Figure 1, **4**) which is actively transported into tumor cells by the sodium/D-glucose cotransporter SGLT3 (SAAT1).³⁸ While the specific glycosylation of **1** has presented analogs which display slight improvements in a perceived therapeutic index (slightly improved in vitro potency and subtle reductions of in vivo peripheral toxicity), the analogs synthesized to date have been restricted to the use of D-glucose or D-galactose-based sugars.^{39–46}

To systematically assess the impact of glycosylation and the corresponding chemoselective linker upon the anticancer activity/selectivity of **1**, herein we report the synthesis and anticancer activities of a 54-member library of **1**-neoglycosides. Several analogs with increased in vitro anticancer activity were identified, most notably D-threoside **60** (NSC 748747) which displayed much broader tumor specificity and increases in potency of up to 15-fold compared to **1** – representing the most active chlorambucil glycoside reported to date. Representative sugars identified as hits in the context of *N*-methoxyamino-substituted **1**, were subsequently conjugated via *N*-acyl hydrazine- and *N*-hydroxyamine-based strategies and the products characterized and evaluated for anticancer activity. Analysis of these second generation neoglycoside analogs revealed sugar-dependent partitioning among open and closed-ring neoglycosides and corresponding sugar-dependent variant biological activity. Cumulatively, this study represents the first neoglycorandomization of a synthetic drug and expands our understanding of the impact of sugar structure upon product distribution/equilibria in the context of *N*-alkoxyamino-, *N*-hydroxyamino- and *N*-acyl hydrazine-based chemoselective glycosylation.

Results and Discussion

Chlorambucil *N*-alkoxyamino-based neoglycorandomization (Scheme 1)

Chlorambucil was converted to the Weinreb amide **6** using the EDAC coupling agent in excellent yield (97%) and then selectively reduced to the corresponding aldehyde with LAH. Compound **7** was subsequently condensed with methoxyamine•HCl in the presence of organic base providing a mixture of *E*- and *Z*-oximes (**8**). Reduction of the carbon-nitrogen double bond was accomplished with $\text{BH}_3\cdot\text{Et}_3\text{N}$ in a manner similar to that for previously described neoaglycons.^{20–21–23} This convenient strategy yielded neoaglycon **9** in four steps with an overall yield of 37% and required only a single chromatographic purification. While standard neoglycosylation conditions (3/1 DMF/HOAc)^{9–22} provided a slightly greater yield (68%) of neo-D-ribose **53** in a pilot reaction, the use of MeOH with a small molar excess of HOAc (i.e., 1.5 equivalents) as an acidic proton source was sufficient for the

^aAbbreviations: $\text{BH}_3\cdot\text{Et}_3\text{N}$, borane-triethylamine complex; CLL, chronic lymphocytic leukemia; CNS, central nervous system; DIC, *N,N*-diisopropylcarbodiimide; DMAP, 4-(*N,N*-dimethylamino)pyridine; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GI50, growth inhibitory concentration for 50% of the cell population under study; GLUT1, glucose transporter 1; LAH, lithium aluminum hydride; NMM, *N*-methylmorpholine; SAAT1, sodium/amino acid transporter 1; SGLT3, sodium/glucose transporter 3; SPE, solid phase extraction; THF, tetrahydrofuran.

reaction to proceed in an equivalent amount of time (54% yield, Table S1, Supporting Information) and also simplified subsequent solvent evacuation. Using these optimized conditions (90 μ M **9**, 2 equiv. sugar, 40 °C, 1.5 eq. HOAc in MeOH) a 54-member library of neoglycosides (**10–63**, see Figure S1, Supporting Information) was synthesized with an average isolated yield of 63% wherein high-throughput solid phase extraction provided an average purity of 92.9% (see Table S3, Supporting Information). Product yields paralleled the overall reactivity trend: tetroses (79 $\pm$ 10%) > deoxy sugars (69 $\pm$ 6%) > pentoses (62 $\pm$ 7%) > hexoses (56 $\pm$ 5%) – likely reflecting the general reducing sugar solubility in the selected solvent system. Unlike prior neoglycorandomized libraries,^{9–24} a preference for β -anomer formation of chlorambucil neoglycosides was generally less predominate. Rather, a strong 1,2-trans relationship was typically observed (Table S2, Supporting Information).

Anticancer activity of chlorambucil *N*-alkoxyamino-based neoglycosides

The antiproliferative activity (i.e., GI₅₀) of neoglycosides **10–63** were evaluated using a ten-member panel of human carcinomas from various lung, colorectal, liver, breast, prostate, CNS, and ovarian cell lines with **1** and neoaglycon **9** as comparators (see Figure 2). Based upon this study, aglycon **9** displayed slightly better GI₅₀ values (1.1–4.4 μ M) compared to the parent drug **1** (5.0–11.0 μ M) and the vast majority of the library members had an average GI₅₀ also comparable to or lower than chlorambucil (see Table S5, Supporting Information). Out of the 54 analogs, 19 possessed GI₅₀s in the high nanomolar range in at least one cell line, six of which displayed GI₅₀s in the high nanomolar range in three or more lines. Of this latter set, two neoglycosides (*D*-glucuronolactonide **39** and *D*-threoside **60**, see Figure 3) were identified as the two most potent neoglycosides. In comparison to the parent **1**, **39** displayed a 6-fold improvement in average growth inhibition across the cell panel, with the most sensitive cell line being SF268 glioblastoma (12-fold improved). Likewise, *D*-threoside **60** presented an average elevated potency of 8-fold over **1**, with 12-, 13-, and 15-fold improved activities toward HT29 (colorectal), H1299 (lung), and HCT15 (colorectal) cancers, respectively, over the parent drug.

The activity assessment described above revealed the following general structure-activity relationships. First, sugars that favor furanosyl-derived neoglycosides (e.g., threosides **60** & **61**, glucuronolactonide **39**, arabinosides **14** & **15**, lyxosides **41** & **42**, and xylosides **62** & **63**) led to the greatest improvements in anticancer activity. Second, relative configuration within this furanoside-derived neoglycoside series influenced potency. Specifically, those tetroses or pentoses with a 2,3-*trans* dihydroxy orientation [e.g., *D*-threoside **60** (2*S*,3*R*), *L*-threoside **61** (2*R*,3*S*), and **39** (*D*-2*S*,3*R*)] were generally more potent than those with a corresponding 2,3-*cis* configuration [e.g., *D*-erythroside **19** (2*R*,3*R*), *D*-riboside **53** (2*R*,3*R*), and *L*-riboside **54** (2*S*,3*S*)] while *D*- and *L*-saccharide enantiomers of this furanoside-derived group were found to have similar values. Third, among neohexosides, a 3*R* hydroxyl group (i.e., axial in the chair conformation) enhances selectivity toward lung (H1299) and colon (HCT-15) cancer cell lines (e.g., allosides **10** & **11**, altrosides **12** & **13**, and *L*-guloside **40**). Finally, most neoglycosides deriving from sugars known to be GLUT substrates and mediate GLUT-dependent uptake of conjugates (e.g., *D*-glucoside **28** or 3-methoxy-*D*-glucoside **33**)^{47–50} were not among the most active and/or selective hits identified. Additionally, comparison of the anomeric composition of either the most active neoglycosides or the library as a whole to the inhibitory data does not reveal that a distinct correlation exists between anomers and activity. To illustrate using the antiproliferative neo-*D*-pentosides (the two with the most extreme anomeric biases), *D*-arabinoside **14** (α/β 19/1) and *D*-xyloside **62** (β only) have similar GI₅₀s in nine of the ten cell lines, not providing any distinction between the two anomers and overall impact on growth inhibition.

Chlorambucil *N*-hydroxyamino-based neoglycosylation (Scheme 2)

Given the clear impact of chlorambucil neoglycosylation upon anticancer activity, we subsequently set out to examine the specific contribution of the neoglycoside handle. Similar to alkoxyamino-based chemoselective neoglycosylation,^{9–24} previous studies have revealed hydroxyamines^{51–53} and hydrazides^{54–59} to also provide the corresponding closed-ring glycosides. Additionally, we envisioned that use of a hydroxyamino handle may allow for additional modification at the hydroxyl group, providing a facile means for further diversification.²³ To create the two modified aglycons, only slight changes in the synthesis were required. In a fashion similar to the procedure described in Scheme 1, aldehyde **7** was combined with hydroxyamine HCl to form a mixture of *E*- and *Z*-oximes (**64**) that were reduced with BH₃•Et₃N complex. As with the synthesis described in Scheme 1, only aglycon **65** required column chromatography for purification in the four-step process from **1** (Scheme 2, 44% overall yield). Hydrazide formation (**71**) was achieved by reacting the *N*-hydroxysuccinimidyl ester of **1** with hydrazine in the presence of DMAP in pyridine. Using the previously described neoglycosylation conditions (see Scheme 1), the focused hydroxyamine (**66–70**) and hydrazide (**72–75**) neoglycosyl sets were synthesized using sugars identified as hits from the alkoxyamino-based series **10–63** – specifically: *D*-glucuronolactone and *D*-threose, which had superior performance throughout the panel; *D*-xylose, the most active of the pentoses; and *D*-fucose, which displayed the most pronounced selectivity toward one tumor line (HCT-15 colorectal).

NMR analysis of the **66–70** neoglycosides revealed the desired closed-ring α - and β -anomeric forms were usually in equilibrium with an open-chain imine isomer. Evidence for this was based upon the observed ¹H NMR chemical shift of the H1 (doublets) and H2 (doublets of doublets) protons at 7.2–7.5 and 5.1–5.0 ppm, respectively, indicating the presence of an iminyl double-bond. Such nitrone formation is well-precedented for the condensation of aldehydes and monosubstituted hydroxyamines.⁶⁰ However, in the context of glycoside formation, only a handful of such glycosides have been reported, comprised of protected saccharides and small *N*-alkyl-*N*-hydroxyamines.^{51–53} From the current study, the nature of the sugar influences the thermodynamic equilibrium and thereby, product distribution. Specifically, the *D*-fucoside-derived **66** predominately adopted the closed-ring isomer (67%), *D*-glucuronolactone (**67**) and *D*-ribose (**68**) favored the open-chain nitrone (71% and 50%, respectively), while *D*-threose (**70**) led solely to nitrone (Figure 4; Table S3, Supporting Information). These equilibria could be shifted upon nitrone modification. For example, peracetylation of neoriboside **68** using acetic anhydride and DMAP in THF promoted ring closure based upon ¹H NMR (Figure 5) and ESI-MS analysis. Deacetylation of the peracetate **69** in base reestablished the mixture of isomers, demonstrating that changing the electronics of the nitrone oxygen will promote ring opening and closure, as will the type of ligated sugar. Interestingly, the anomeric ratio also changed, moving from a 1/1 ratio as the nitrone to 2/1 as the tetraacetate, contrasting from the 1/3 α/β ratio of methoxyamine *D*-ribose (**53**).

Chlorambucil *N*-acyl hydrazine-based neoglycosylation (Scheme 2)

A conformational study of sugar acetylhydrazides by Bendiak indicated that natural hexose and pentose (e.g., *D*-Glc, *D*-Gal, *D*-Xyl, etc.) analogs formed pyranosides, as evidenced by ¹H NMR.⁵⁴ Similar findings were reported for mono- and oligosaccharide chemoselective ligations with biotinyl,⁵⁵ long-chain acyl,⁵⁶ peptidyl,⁵⁷ and adipyl hydrazides.⁵⁹ In contrast, the current chlorambucil study revealed a trend that mirrored that which was observed for the **66–70** subset. Specifically, *D*-fucoside **72** was formed only as the cyclic compound (67% yield), *D*-glucuronolactonide **73** and *D*-xyloside **75** were a mixture of the open and closed conformers (46% and 47%, respectively), and *D*-threoside **74** adopted the open-chain imine (86%). These results indicate that closure of the glycoside may not only be

dependent on the type of sugar but possibly the nature of the aglycon as well. It was also notable that only the β -anomer of the closed-rings was observed while the hydrazyl imines were isolated as mixtures of *E*- and *Z*-isomers (Figure 6).

Anticancer activity of chlorambucil *N*-hydroxyamino- and *N*-acyl hydrazine-based neoglycosides

Neoglycosides **66–70**, **72–75**, and their respective aglycons, **65** and **71**, were assayed for antiproliferative activity using the same cell lines as **10–63**. In general, the alternate handle glycosides did not perform as well as their methoxyamine analogs with the most notable difference being between the **66–70** and **72–75** analogs. While some of the hydroxyamine compounds produced comparable inhibitory responses to the methoxyamine group, **72–75** had universally diminished anticancer properties over **10–63** with average GI₅₀ values 5- to 13-fold greater across the ten-member panel. **66–70** and **72–75** were also found to have GI₅₀ values up to 6-fold larger than the corresponding aglycons, though aglycons **65** and **71** were of similar activity to **9** (Table S5, Supporting Information).

Two exceptions to this overall trend, *D*-riboside **68** and its peracetylated variant **69**, were 2- to 5-fold more active than the alkoxyamino-based **53** in six of the ten cell lines (0.53–1.3 μ M vs. 1.8–5.9 μ M, respectively). **68** and **69** are also notable as among the most active (i.e., mid- to high-nanomolar range) neoglycosides against the SKOV3 ovarian cancer line (see Figure 7). These data suggest the type of neoglycoside handle employed greatly influences desired activity. When taken into account that aglycons **9**, **65**, and **71** are of similar potency, the variation in activity between the methoxyamino-, hydroxyamino-, and acyl hydrazine-based neoglycosides implicates the nature of the glycosidic bond to be the most significant contributor wherein a prevalence of the acyclic nitron or imine conjugate led to a reduction in potency.

Conclusions

In summary, this study revealed a facile four step process, which could be conducted on gram scale in less than two hours of reaction time and required only a single chromatographic separation, to modify the drug chlorambucil (**1**) for chemoselective glycosylation. While prior syntheses of **1**-glycoconjugates focused upon the use of typical metabolic sugars designed to enhance sugar-mediated uptake and led to modest overall improvements compared to **1**,^{39,40} the current study revealed anticancer potency optimization was best accomplished via conjugation with novel non-metabolic sugars – culminating in the discovery of *D*-threoside **60** as the most active chlorambucil glycoside reported to date. The discovery of **60** opens the door to a series of new questions relating to the precise mechanism(s) of improvement – including among the many possibilities: *i*) modulation of uptake (via novel targeting of known transporters and/or even raising the possibility of new sugar transport/receptor mediated-processes); *ii*) intracellular stabilization of the alkylating reagent (basically extending the intracellular T_{1/2}); *iii*) enhancing the productive agent-DNA interactions (*e.g.*, DNA affinity and/or specificity); and/or even *iv*) alternative targeting of the active species (*e.g.*, RNA, proteins, and/or membrane targets). While the specific mechanism(s) remain to be elucidated, it is important to note that precedent does exist for **1** glucosylation to alter the mechanism of cellular uptake.^{34,42}

In addition to the lead discovery aspect of this project, it is also important to note that the corresponding in-depth product distribution analysis among *N*-alkoxyamino-, *N*-acyl hydrazine- and *N*-hydroxyamino-based neoglycosylation reactions revealed sugar-dependent partitioning among open and closed-ring neoglycosides in the latter two cases. Thus, this cumulative study also sheds new light (and a potential note of caution) on an under-appreciated chemical variability of glycoconjugates generated via the *N*-acyl hydrazine- and

N-hydroxyamine-based chemoselective glycosylation methods more commonly applied in glycobiology.¹⁸

Experimental Section

Materials and General Methods

Mass spectrometric data were obtained on either a Waters (Milford, MA) LCT time-of-flight spectrometer for electrospray ionization (ESI) or a Varian ProMALDI (Palo Alto, CA) Fourier-transform ion cyclotron resonance mass spectrometer (FTICR) equipped with a 7.0 Tesla actively-shielded superconducting magnet and a Nd-YAG laser. NMR spectra were obtained on a Varian ^{Unity}Inova 500 MHz instrument (Palo Alto, CA) using 99.8% CDCl₃ with 0.05% v/v TMS or 99.8% CD₃OD in ampoules. ¹H and ¹³C chemical shifts were referenced to TMS (for CDCl₃) or nondeuterated solvent (for CD₃OD). Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), qui (quintet), m (multiplet), and br (broad). Chemical shift assignments for anomeric mixtures, where possible, are noted as α or β with the atom responsible for the shift. ¹H NMR characterization was supplemented with gCOSY for all neoglycoside library members as well as ¹³C and gHSQC for pilot reactions and alternate handle compounds. Tetrahydrofuran was dried using a column of activated alumina. All other solvents were used as provided by the supplier. Reagents were obtained from Aldrich or Sigma and were used as received. Flash chromatography was performed using 40–63 μm particle sized silica gel. Thin layer chromatography was performed on aluminum-backed, 254 nm UV-active plates with a silica gel particle size of 60 μm. Library purity was assessed by reverse phase HPLC using a Varian (Walnut Creek, CA) ProStar unit with a Phenomenex (Torrance, CA) Luna C18 4.6 × 250 mm column running a H₂O/MeCN 90/10 to 10/90 gradient over 13 m, followed by a 5 m isocratic flow, at a rate of 1.0 mL/m., A₂₅₄ detection. Purity of the neoglycosides and aglycons was assessed to be greater than 95%, unless specified otherwise (see Table S4, Supporting Information).

N,O-Dimethyl 4-(4-*N',N'*-bis(2-chloroethyl)amino)phenylbutanamide (6)

Chlorambucil (**1**, 516 mg, 1.70 mmol) was dissolved in CH₂Cl₂ (15 mL) and cooled to 0 °C before adding *N,O*-dimethylhydroxylamine HCl (181 mg, 1.86 mmol) and *N*-methylmorpholine (200 μL, 1.82 mmol). The coupling agent EDAC (347 mg, 1.81 mmol) was then added to the reaction slowly over 5 min to ensure dissolution. After 60 min, the reaction was quenched with 5% HCl (20 mL) and the acidic layer extracted with CH₂Cl₂ (20 mL). The organic layers were combined, washed with sat. aq. NaHCO₃ (20 mL) and the basic layer extracted with CH₂Cl₂ (20 mL). After combining the organic layers, washing with brine (20 mL), and drying with Na₂SO₄, solvent removal yielded a colorless oil (571 mg, 97%, R_f = 0.23 EtOAc:Hex 1:2) that was used without further purification. ¹H NMR (CDCl₃, 500 MHz) δ 7.09 (d, *J* = 8.8 Hz, 2 H), 6.62 (d, *J* = 8.8 Hz, 2 H), 3.71–3.65 (m, 4 H), 3.64 (s, 3 H), 3.62–3.59 (m, 4 H), 3.17 (s, 3 H), 2.58 (t, *J* = 7.7 Hz, 2 H), 2.44 (t, *J* = 7.0 Hz, 2 H), 1.95–1.88 (m, 2 H); ¹³C NMR (CDCl₃, 125 MHz) δ 144.37, 131.17, 129.80, 112.31, 61.30, 53.74, 40.69, 34.33, 32.29, 31.35, 26.44; HRMS (ESI) *m/z* for C₁₆H₂₅Cl₂N₂O₂ ([M+H]⁺) 347.1295, calc. 347.1288.

4-(4-*N',N'*-bis(2-chloroethyl)amino)phenylbutanal (7)

Weinreb amide **6** (571 mg, 1.64 mmol) was dissolved in anhydrous THF (8 mL) under Ar and cooled to 0 °C. A suspension of lithium aluminum hydride in anhydrous THF (1.0 M, 1 mL) was added in one aliquot. After 5 min, the reaction was quenched with sat. aq. KHSO₄ (5 mL) followed by deionized water (5 mL). Extraction of the aldehyde was performed with Et₂O (2 × 15 mL) and the organic layer was dried with Na₂SO₄. Solvent evaporation provided a yellowish oil (334 mg, 70%, R_f = 0.65 EtOAc:Hex 1:2). ¹H NMR (CDCl₃, 500 MHz) δ 9.80 (s, 1 H), 7.13 (d, *J* = 8.8 Hz, 2 H), 6.70 (d, *J* = 8.8 Hz, 2 H), 3.77–3.74 (m, 4

H), 3.69–3.66 (m, 4 H), 2.63 (t, $J = 7.6$ Hz, 2 H), 2.49 (td, $J = 7.3, 1.5$ Hz, 2 H), 2.00–1.94 (m, 2 H); ^{13}C NMR (CDCl_3 , 125 MHz) δ 202.42, 144.46, 130.37, 129.69, 112.23, 53.56, 43.16, 40.60, 33.91, 23.93; HRMS (MALDI) m/z for $\text{C}_{14}\text{H}_{20}\text{Cl}_2\text{NO}$ ($[\text{M}+\text{H}]^+$) 288.09091, calc. 288.09165.

***N*-Methoxy-4-(4-*N'*,*N'*-bis(2-chloroethyl)amino)phenylbutanimine (8)**

Aldehyde **6** (334 mg, 1.16 mmol) was dissolved in absolute EtOH (10 mL) followed by the addition of $\text{MeONH}_2\cdot\text{HCl}$ (247 mg, 2.95 mmol) and Et_3N (400 μL , 2.87 mmol). After 35 min, the solvent was removed in vacuo, providing the crude product as a white crystalline solid. The material was suspended in EtOAc:Hex 1:7 (50 mL) and filtered through a silica gel plug, which was flushed with solvent (400 mL). The purified imine was provided as a colorless oil containing a mixture of *E*- and *Z*-isomers (308 mg, 84%, $R_{\text{fA}} = 0.51$ $R_{\text{fB}} = 0.43$ EtOAc:Hex 1:7). ^1H NMR (CDCl_3 , 500 MHz) δ 7.36 (t, $J = 6.2$ Hz, 1 H), 7.05 (d, $J = 8.8$ Hz, 2 H), 6.61 (d, $J = 8.8$ Hz, 2 H), 3.80 (s, 3 H), 3.69–3.65 (m, 4 H), 3.61–3.57 (m, 4 H), 2.55 (t, $J = 7.7$ Hz, 2 H), 2.18 (q, $J = 7.7$ Hz, 2 H), 1.75 (qui, $J = 7.7$ Hz, 2 H); ^{13}C NMR (CDCl_3 , 125 MHz) δ 150.58, 144.38, 130.87, 129.74, 112.28, 61.27, 53.67, 40.61, 34.14, 29.03, 28.72; HRMS (ESI) m/z for $\text{C}_{15}\text{H}_{23}\text{Cl}_2\text{N}_2\text{O}$ ($[\text{M}+\text{H}]^+$) 317.1187, calc. 317.1182.

***N*-Methoxy-4-(4-*N'*,*N'*-bis(2-chloroethyl)amino)phenylbutylamine (9)**

Imine **8** (308 mg, 0.971 mmol) was dissolved in absolute EtOH (15 mL), cooled to 0 °C, and the reducing agent $\text{BH}_3\cdot\text{Et}_3\text{N}$ (710 μL , 4.83 mmol) was added in one aliquot. Concentrated $\text{HCl}:\text{EtOH}$ 1:1 (800 μL) was then dripped in slowly over 5 min. The reaction was quenched with sat. aq. NaHCO_3 (5 mL), 5 min after the acid solution was completely added, resulting in a white slurry. The organic solvent was removed under reduced pressure then more NaHCO_3 (5 mL) solution was added. The aqueous material was extracted with CH_2Cl_2 (3×20 mL) and dried with Na_2SO_4 . The crude oil was purified by flash chromatography (SiO_2 , EtOAc:Hex 1:2) providing the aglycon as a colorless oil (200 mg, 65%, $R_{\text{f}} = 0.37$ EtOAc:Hex 1:2). ^1H NMR (CDCl_3 , 500 MHz) δ 7.05 (d, $J = 8.6$ Hz, 2 H), 6.60 (d, $J = 8.7$ Hz, 2 H), 5.50 (s br, 1 H), 3.69–3.65 (m, 4 H), 3.61–3.57 (m, 4 H), 3.50 (s, 3 H), 2.91 (t, $J = 7.1$ Hz, 2 H), 2.53 (t, $J = 7.5$ Hz, 2 H), 1.61–1.58 (m, 2 H), 1.55–1.49 (m, 2 H); ^{13}C NMR (CDCl_3 , 125 MHz) δ 144.20, 131.63, 129.62, 112.22, 61.80, 53.65, 51.80, 40.61, 34.66, 29.32, 26.98; HRMS (ESI) m/z for $\text{C}_{15}\text{H}_{25}\text{Cl}_2\text{N}_2\text{O}$ ($[\text{M}+\text{H}]^+$) 319.1334, calc. 319.1339.

***N*-Hydroxy-4-(4-*N'*,*N'*-bis(2-chloroethyl)amino)phenylbutanimine (64)**

Aldehyde **7** (442 mg, 1.53 mmol) was dissolved in absolute EtOH (10 mL) followed by the addition of $\text{HONH}_2\cdot\text{HCl}$ (269 mg, 3.87 mmol) and Et_3N (550 μL , 3.95 mmol). After 25 min, the solvent was removed in vacuo, providing the crude product as a white flaky solid. The material was purified by column chromatography (SiO_2 , EtOAc:Hex 1:2), which yielded the imine as a colorless mixture of *E*- and *Z*-isomers (455 mg, 98%, $R_{\text{fA}} = 0.53$ $R_{\text{fB}} = 0.45$ EtOAc:Hex 1:2). ^1H NMR (CDCl_3 , 500 MHz) δ 7.29 (t, $J = 6.3$ Hz, 1 H), 7.06 (d, $J = 8.8$ Hz, 2 H), 6.61 (d, $J = 8.8$ Hz, 2 H), 3.68–3.65 (m, 4 H), 3.61–3.58 (m, 4 H), 2.55 (t, $J = 7.7$ Hz, 2 H), 2.15 (q, $J = 7.7$ Hz, 2 H), 1.75 (qui, $J = 7.7$ Hz, 2 H); ^{13}C NMR (CDCl_3 , 125 MHz) δ 147.98, 144.45, 131.01, 129.69, 112.22, 53.76, 40.59, 33.23, 29.10, 28.73; HRMS (ESI) m/z for $\text{C}_{14}\text{H}_{21}\text{Cl}_2\text{N}_2\text{O}$ ($[\text{M}+\text{H}]^+$) 303.1019, calc. 303.1025.

***N*-Hydroxy-4-(4-*N'*,*N'*-bis(2-chloroethyl)amino)phenylbutylamine (65)**

Imine **64** (455 mg, 1.50 mmol) was dissolved in absolute EtOH (10 mL), cooled to 0 °C, and the reducing agent $\text{BH}_3\cdot\text{Et}_3\text{N}$ (1.1 mL, 7.5 mmol) was added in one aliquot. Concentrated $\text{HCl}:\text{EtOH}$ 1:1 (1.26 μL) was then dripped in slowly over 5 min. The reaction was quenched with sat. aq. NaHCO_3 (5 mL), 5 min after the acid solution was completely added, resulting in a white slurry. The organic solvent was removed under reduced pressure then more

NaHCO₃ (5 mL) solution was added. The aqueous material was extracted with CH₂Cl₂ (4 × 25 mL) and dried with Na₂SO₄. The crude oil was purified by column chromatography (SiO₂, MeOH:CH₂Cl₂ 2:98 to 5:95) providing the aglycon as a colorless oil (302 mg, 66%, R_f = 0.36 MeOH:CH₂Cl₂ 5:95). ¹H NMR (CDCl₃, 500 MHz) δ 7.05 (d, *J* = 8.7 Hz, 2 H), 6.61 (d, *J* = 8.7 Hz, 2 H), 5.23 (s br, 2 H), 3.70–3.64 (m, 4 H), 3.63–3.57 (m, 4 H), 3.40 (s, 2 H), 2.58–2.51 (m, 2 H), 1.96–1.84 (m, 2 H), 1.67–1.55 (m, 2 H); ¹³C NMR (CDCl₃, 125 MHz) δ 144.60, 131.08, 129.84, 112.47, 53.81, 50.43, 40.88, 34.58, 29.00, 27.16; HRMS (ESI) *m/z* for C₁₄H₂₃Cl₂N₂O ([M+H]⁺) 305.1175, calc. 305.1182.

4-(4-*N',N'*-bis(2-chloroethyl)amino)phenylbutanoic hydrazide (71)

Chlorambucil (**1**, 319 mg, 1.05 mmol) was dissolved in THF (10 mL), along with *N*-hydroxysuccinimide (132 mg, 1.15 mmol) and DIC (180 μL, 1.16 mmol). The reaction was warmed to 40 °C and stirred for 5.5 h. Hydrazine (40 μL, 1.27 mmol), pyridine (10 μL), and DMAP (5 mg, 0.04 mmol) were then introduced and the reaction proceeded for another 20 min. The solvent was removed in vacuo yielding a yellowish solid, which was suspended in MeOH:CH₂Cl₂ 3:97 and purified by column chromatography (SiO₂, MeOH:CH₂Cl₂ 3:97). The hydrazide product was collected as an opaque oil that solidified at –20 °C to a white amorphous solid (285 mg, 85% R_f = 0.39 MeOH:CH₂Cl₂ 5:95). ¹H NMR (CDCl₃, 500 MHz) δ 7.31 (s br, 1 H), 7.05 (d, *J* = 8.7 Hz, 2 H), 6.61 (d, *J* = 8.7 Hz, 2 H), 3.90 (s br, 2 H), 3.71–3.66 (m, 4 H), 3.63–3.58 (m, 4 H), 2.54 (t, *J* = 7.6 Hz, 2 H), 2.17–2.13 (m, 2 H), 1.95–1.89 (m, 2 H); ¹³C NMR (CDCl₃, 125 MHz) δ 173.80, 144.46, 130.47, 129.72, 112.26, 53.62, 40.66, 40.88, 34.10, 33.77, 27.19; HRMS (ESI) *m/z* for C₁₄H₂₂Cl₂N₃O ([M+H]⁺) 318.1140, calc. 318.1134.

General procedure for neoglycoside library synthesis, purification, and characterization

Aglycons **9**, **65**, or **71** (typically 0.12–0.16 mmol) were added to 1 dram vials along with stir fleas and dissolved in MeOH such that the aglycon was at a concentration of 90–100 mM. Glacial acetic acid (1.5 eq.) was introduced, reducing sugars (2 eq.) were added, the vials capped, and the vessels placed on a heating block/stir plate to react at 40 °C for 3–48 h. The vial caps were removed and the solvent evaporated by Speedvac apparatus (55 °C, 3 h). Crude neoglycosides were suspended in CH₂Cl₂ (200 μL) using a vortex mixer then loaded onto 2000 mg silica gel solid phase extraction (SPE) columns (Alltech, Deerfield, IL) that were prewashed with MeOH/CH₂Cl₂ 2/98. The SPEs were eluted using a vacuum manifold, collecting fractions with a volume of approximately 2 mL. After the initial two fractions were obtained, eluting any unreacted aglycon or relatively nonpolar material, the following step gradients were used: MeOH/CH₂Cl₂ 5/95 for tetroses, pentoses, and substituted hexoses; MeOH/CH₂Cl₂ 10/90 for hexoses; MeOH/CHCl₃ 15/85 for disaccharides; MeOH/CHCl₃ 20/80 for glycuronosides. For polyprotected saccharides, a gradient of EtOAc/Hex 1/6 to 1/5 was used. Typically, all neoglycoside was eluted by the tenth or eleventh fraction, leaving unreacted sugar on the column. The fractions containing pure product were identified by TLC using UV light (254 nm) and *p*-anisaldehyde stain, then combined and dried. Library members were characterized by ¹H and gCOSY NMR as well as either high-resolution ESI or MALDI mass spectrometry (see Tables S2 and S3, Supporting Information). Anomeric ratios were obtained by comparison of anomeric proton integration (see Tables S2 and S3, Supporting Information).

N-Methoxy-4-(4-*N',N'*-bis(2-chloroethyl)amino)phenylbutylamino-*D*-ribose (53)

Aglycon **9** (40 mg, 0.13 mmol) was placed into a 1 dram vial, dissolved in MeOH (1.34 mL), and mixed with glacial acetic acid (10.7 μL). After adding *D*-ribose (57 mg, 0.38 mmol), the reaction was capped, warmed to 40 °C and allowed to stir for 3 h. Solvent was subsequently removed in vacuo and the resulting crude solid suspended in MeOH:CH₂Cl₂ 2:98 (250 μL) by vortex mixer. The mixture was purified by SPE (SiO₂, MeOH:CH₂Cl₂

2:98 to 5:95), providing the white solid neoglycoside as a mixture of anomers (31 mg, 54%, $R_f = 0.34$ MeOH:CH₂Cl₂ 5:95; α : β 1:3). ¹H NMR (CD₃OD, 500 MHz) δ 7.08 (d, $J = 8.7$ Hz, 2 H), 6.68 (d, $J = 8.7$ Hz, 2 H), 4.56 (d, $J = 3.4$ Hz, 0.25 H, α -H1'), 4.27 (d, $J = 8.7$ Hz, 0.75 H, β -H1'), 4.15 (dd, $J = 5.4, 3.5$ Hz, 0.25 H, α -H2'), 4.12 (s br, 0.75 H, β -H3'), 3.99 (t, $J = 5.6$ Hz, 0.25 H, α -H3'), 3.90–3.86 (m, 0.25, α -H4'), 3.74–3.68 (m, 5 H, α -H5_A' + β -H5_A'), 3.68–3.60 (m, 6.5 H, α -H5_B' + β -H2' + β -H4' + β -H5_B'), 3.58 (s, 3 H), 3.05–2.98 (m, 0.75 H, β -H2_A), 2.97–2.92 (m, 0.25 H, α -H2_A), 2.86–2.77 (m, 1 H, H2_B), 2.56 (t, $J = 7.2$ Hz, 2 H), 1.71–1.56 (m, 4 H); ¹³C NMR (CD₃OD, 125 MHz) δ 145.86, 132.73, 130.66, 113.61, 100.76 (α -C1'), 91.14 (β -C1'), 84.50 (α -C4'), 73.16 (α -C2'), 72.52 (α -C3'), 72.46 (β -C3'), 68.94 (β -C2'), 68.65 (β -C4'), 65.94 (β -C5'), 64.21 (α -C5'), 62.82, 54.76 (α -C2), 54.68, 54.05 (β -C2), 41.82, 35.76, 30.70, 28.12; HRMS (ESI) m/z for C₂₀H₃₂Cl₂N₂NaO₅ ([M+Na]⁺) 473.1581, calc. 473.1587.

***N*-Methoxy-4-(4-*N'*,*N'*-bis(2-chloroethyl)amino)phenylbutylamino-L-ribose (54)**

Using the same procedure as **53**, **9** (52 mg, 0.16 mmol) combined with L-ribose (48 mg, 0.32 mmol), yielded the anomeric mixture as a white solid (40 mg, 54%, $R_f = 0.34$ MeOH:CH₂Cl₂ 5:95; α : β 1:3). ¹H NMR (CD₃OD, 500 MHz) δ 7.05 (d, $J = 8.6$ Hz, 2 H), 6.67 (d, $J = 8.6$ Hz, 2 H), 4.52 (d, $J = 3.4$ Hz, 0.25 H, α -H1'), 4.25 (d, $J = 8.7$ Hz, 0.75 H, β -H1'), 4.11 (dd, $J = 5.4, 3.5$ Hz, 0.25 H, α -H2'), 4.08 (s br, 0.75 H, β -H3'), 3.95 (t, $J = 5.5$ Hz, 0.25 H, α -H3'), 3.87–3.83 (m, 0.25, α -H4'), 3.71–3.67 (m, 5 H, α -H5_A' + β -H5_A'), 3.65–3.61 (m, 5.75 H, α -H5_B' + β -H4' + β -H5_B'), 3.60–3.59 (m, 0.75 H, β -H2'), 3.55 (s, 3 H), 3.00–2.95 (m, 0.75 H, β -H2_A), 2.94–2.89 (m, 0.25 H, α -H2_A), 2.81–2.76 (m, 1 H, H2_B), 2.53 (t, $J = 7.5$ Hz, 2 H), 1.66–1.55 (m, 4 H); (CD₃OD, 125 MHz) δ 145.96, 132.83, 130.69, 113.70, 100.85 (α -C1'), 91.20 (β -C1'), 84.59 (α -C4'), 73.24 (α -C2'), 72.61 (α -C3'), 72.56 (β -C3'), 69.02 (β -C2'), 68.74 (β -C4'), 66.00 (β -C5'), 64.28 (α -C5'), 62.81, 54.78 (α -C2), 54.76, 54.07 (β -C2), 41.86, 35.80, 30.75, 28.15; HRMS (ESI) m/z for C₂₀H₃₂Cl₂N₂NaO₅ ([M+Na]⁺) 473.1579, calc. 473.1581.

***N*-Hydroxy-4-(4-*N'*,*N'*-bis(2-chloroethyl)amino)phenylbutylamino- α -fucoside (66)**

Using aglycon **65** (63 mg, 0.21 mmol), the mixture of compounds was yielded as a white solid (54 mg, 58%, $R_f = 0.11$ MeOH:CH₂Cl₂ 5:95; α : β :nitrone 2.7:1:1.8). ¹H NMR (CD₃OD, 500 MHz) δ 7.25 (d, $J = 5.8$ Hz, 0.33 H, nitrone-H1'), 7.10–7.07 (m, 2 H), 6.70–6.67 (m, 2 H), 5.06 (dd, $J = 5.8, 2.1$ Hz, 0.33 H, nitrone-H2'), 4.40 (d, $J = 4.9$ Hz, 0.18 H, α -H1'), 4.30 (dd, $J = 5.8, 4.9$ Hz, 0.18 H, α -H2'), 4.08 (dd, $J = 6.6, 1.8$ Hz, 0.33 H, nitrone-H5'), 3.96 (dd, $J = 7.5, 5.8$ Hz, 0.18 H, α -H3'), 3.89 (dd, $J = 8.7, 2.1$ Hz, 0.33 H, nitrone-H3'), 3.86–3.79 (m, 1.33 H, nitrone-H2 + α -H5' + β -H1'), 3.73–3.70 (m, 4.49 H, β -H2'), 3.67–3.63 (m, 4.18 H, α -H4'), 3.61–3.58 (m, 0.98 H, β -H4' + β -H5'), 3.53–3.50 (m, 0.49 H, β -H3'), 3.48 (dd, $J = 8.7, 1.8$ Hz, 0.33 H, nitrone-H4'), 3.11–3.06 (m, 0.49 H, β -H2_A), 3.05–3.01 (m, 0.18 H, α -H2_A), 2.79–2.75 (m, 0.49 H, β -H2_B), 2.74–2.68 (m, 0.18 H, α -H2_B), 2.60–2.54 (m, 2 H), 1.91–1.87 (m, 0.66 H, nitrone-H3), 1.68–1.63 (m, 3.34 H), 1.30–1.26 (m, 3 H, H6'); ¹³C NMR (CD₃OD, 125 MHz) δ 145.54 (nitrone-C1'), 144.64, 131.68, 129.45, 112.41, 99.46 (α -C1'), 94.89 (β -C1'), 86.70 (β -C2'), 78.52 (α -C2'), 77.32 (α -C3'), 74.99 (β -C3'), 73.68 (nitrone-C4'), 72.48 (β -C5'), 72.36 (β -C4'), 71.32 (nitrone-C3'), 67.76 (α -C5'), 67.38 (α -C4'), 67.26 (nitrone-C2'), 66.21 (nitrone-C5'), 64.69 (nitrone-C2), 54.87 (β -C2), 53.72 (α -C2), 53.49, 40.64, 34.59, 29.36, 28.16 (α / β -C3), 26.97 (nitrone-C3), 18.95 (α / β -C6'), 18.66 (nitrone-C6'); HRMS (MALDI) m/z for C₂₀H₃₂Cl₂N₂NaO₅ ([M+Na]⁺) 473.15911, calc. 473.15805.

***N*-Hydroxy-4-(4-*N'*,*N'*-bis(2-chloroethyl)amino)phenylbutylamino- α -glucurono-6,3-lactonide (67)**

Using aglycon **65** (58 mg, 0.19 mmol), the mixture of compounds was yielded as a colorless syrup (49 mg, 56%, $R_f = 0.28$ MeOH:CH₂Cl₂ 5:95; α : β :nitrone 0:1:2.5). ¹H NMR (CD₃OD,

500 MHz) δ 7.25 (d, J = 6.3 Hz, 0.71 H, nitrone-H1'), 7.10 (d, J = 8.6 Hz, 2 H), 6.70 (d, J = 8.6 Hz, 2 H), 5.11–5.08 (m, 0.71 H, nitrone-H2'), 4.85–4.83 (m, 0.29 H, β -H4'), 4.79 (d, J = 4.4 Hz, 0.29 H, β -H3'), 4.57 (d, J = 2.3 Hz, 0.29 H, β -H1'), 4.54 (d, J = 4.6 Hz, 0.71 H, nitrone-H5'), 4.49 (d, J = 2.0 Hz, 0.29 H, β -H5'), 4.47 (d, J = 2.8 Hz, 0.71 H, nitrone-H3'), 4.46 (d, J = 2.3 Hz, 0.29 H, β -H2'), 4.33 (dd, J = 4.6, 2.8 Hz, 0.71 H, nitrone-H4'), 3.91 (q, J = 7.0 Hz, 1.42 H, nitrone-H2), 3.74–3.71 (m, 4 H), 3.67–3.65 (m, 4 H), 2.99–2.96 (m, 0.29 H, β -H2_A), 2.69–2.64 (m, 0.29 H, β -H2_B), 2.59 (t, J = 7.5 Hz, 1.42 H, nitrone-H5), 2.69–2.64 (dd, J = 7.2, 6.7 Hz, 0.58 H, β -H5), 1.94–1.87 (m, 1.42 H, nitrone-H3), 1.68–1.62 (m, 2.58 H, β -H3 + H4); ¹³C NMR (CD₃OD, 125 MHz) δ 176.39 (C6'), 144.87, 141.51 (nitrone-C1'), 130.76, 129.48, 112.45, 104.94 (β -C1'), 85.74 (β -C3'), 80.64 (nitrone-C3'), 77.57 (β -C4'), 77.24 (β -C2'), 70.90 (nitrone-C5'), 69.97 (nitrone-C4'), 69.65 (β -C5'), 66.13 (nitrone-C2'), 65.02 (nitrone-C2), 55.41 (β -C2), 53.42, 40.66, 34.55 (β -C5), 33.99 (nitrone-C5), 29.15 (β -C3), 28.17, 26.79 (nitrone-C3); HRMS (MALDI) m/z for C₂₀H₂₈Cl₂N₂NaO₆ ([M + Na]⁺) 485.12254, calc. 485.12166.

N-Hydroxy-4-(4-*N'*,*N'*-bis(2-chloroethyl)amino)phenylbutylamino- β -ribose (68)

Using aglycon **65** (50 mg, 0.16 mmol), the product mixture was yielded as a white solid that was visualized as a single spot by TLC (18 mg, 25%, R_f = 0.39 MeOH:CH₂Cl₂ 10:90; α : β :nitrone 1:1:2). ¹H NMR (CD₃OD, 500 MHz) δ 7.17 (d, J = 6.3 Hz, 0.5 H, nitrone-H1'), 7.07 (d, J = 8.7 Hz, 2 H), 6.66 (d, J = 8.7 Hz, 2 H), 4.99 (dd, J = 6.3, 3.5 Hz, 0.5 H, nitrone-H2'), 4.51 (d, J = 2.9 Hz, 0.25 H, α -H1'), 4.18–4.17 (m, 0.25 H, α -H2'), 4.16 (d, J = 8.7 Hz, 0.25 H, β -H1'), 4.12–4.09 (m, 0.5 H, α/β -H4' + β -H2'), 3.89–3.86 (m, 0.5 H, α -H3' + α/β -H5_A'), 3.86–3.85 (m, 1 H, nitrone-H2), 3.84–3.81 (m, 0.25 H, α/β -H5_A'), 3.78 (dd, J = 11.5, 3.5 Hz, 0.5 H, nitrone-H3'), 3.74–3.67 (m, 5 H, nitrone-H5_A' + α/β -H4' + α/β -H5_B'), 3.65–3.58 (m, 5 H, nitrone-H5_B' + α/β -H5_B' + β -H3'), 3.55–3.51 (m, 0.5 H, nitrone-H4'), 3.08–3.02 (m, 0.25 H, α -H2_A), 2.98–2.93 (m, 0.25 H, α -H2_B), 2.74–2.67 (m, 0.5 H, β -H2), 2.57 (t, J = 7.4 Hz, 2 H), 1.92–1.86 (m, 2 H), 1.68–1.60 (m, 2 H); ¹³C NMR (CD₃OD, 125 MHz) δ 144.79, 144.63 (nitrone-C1'), 130.80, 129.52, 112.45, 100.86 (α -C1'), 91.12 (β -C1'), 83.43 (α -C3'), 74.19 (nitrone-C3'), 72.98 (α -C2'), 72.31 (nitrone-C4'), 70.79 (β -C2'), 70.71 (α/β -C4'), 68.16 (nitrone-C2'), 67.88 (β -C3'), 67.55 (α/β -C4'), 64.85 (α/β -C5'), 64.82, 63.58 (nitrone-C5'), 62.59 (α/β -C5'), 53.54, 40.57, 34.09, 28.22, 26.78; HRMS (ESI) m/z for C₁₉H₃₀Cl₂N₂NaO₅ ([M + Na]⁺) 459.1441, calc. 459.1424.

N-Acetoxy-4-(4-*N'*,*N'*-bis(2-chloroethyl)amino)phenylbutylamino- β -ribose peracetate (69)

The β -ribose mixture designated **68** (23 mg, 0.053 mmol) was peracetylated by dissolving in THF (5 mL) and adding acetic anhydride (0.5 mL, 5 mmol), DMAP (1 mg, 0.008 mmol), and Et₃N (0.5 mL, 4 mmol). After 20 min, the solvent was removed in vacuo and the residue was purified by SPE chromatography (SiO₂, EtOAc:Hex 2:3). The product was collected as a mixture of anomers of a colorless oil (32 mg, >99%, R_f β = 0.38 R_f α = 0.32 EtOAc:Hex 2:3; α : β = 2:1). ¹H NMR (CDCl₃, 500 MHz) δ 7.06–7.04 (m, 2 H), 6.61 (d, J = 8.6 Hz, 2 H), 5.62 (s, 0.33 H, β -H3'), 5.27–5.25 (m, 0.67 H, α -H2'), 5.21 (dd, J = 6.1, 5.4 Hz, 0.67 H, α -H3'), 5.07 (dd, J = 9.2, 2.6 Hz, 0.33 H, β -H2'), 5.04–5.01 (m, 0.33 H, β -H4'), 4.89 (d, J = 2.9 Hz, 0.67 H, α -H1'), 4.58 (d, J = 9.2 Hz, 0.33 H, β -H1'), 4.33–4.31 (m, 0.67 H, α -H5_A'), 4.23–4.20 (m, 0.67 H, α -H4'), 4.16 (dd, J = 11.7, 5.4 Hz, 0.67 H, α -H5_B'), 3.97 (dd, J = 10.9, 5.4 Hz, 0.33 H, β -H5_A'), 3.71–3.67 (m, 4.33 H, β -H5_B'), 3.63–3.60 (m, 4 H), 3.20–3.15 (m, 0.33 H, β -H2_A), 3.11–3.06 (m, 0.67 H, α -H2_A), 2.99–2.91 (m, 1 H, α -H2_B + β -H2_B), 2.57–2.47 (m, 2 H), 2.11 (s, 3 H), 2.10 (s, 3 H), 2.09 (s, 3 H), 2.05 (s, 3 H), 1.67–1.46 (m, 4 H); ¹³C NMR (CDCl₃, 125 MHz) δ 170.72, 169.76, 169.51, 169.49, 144.35, 131.49, 129.74, 112.36, 96.56 (α -C1'), 88.60 (β -C1'), 77.85 (α -C4'), 71.99 (α -C2'), 70.58 (α -C3'), 68.93 (β -C3'), 66.47 (β -C4'), 65.72 (β -C2'), 63.55 (α -C5'), 63.21 (β -C5'), 54.06, 53.79, 40.71, 34.55, 29.20, 26.53, 20.93, 20.88, 20.68, 20.65; HRMS (ESI) m/z for C₂₇H₃₈Cl₂N₂NaO₉ ([M + Na]⁺) 627.1859, calc. 627.1847.

N-Hydroxy-4-(4-*N'*,*N'*-bis(2-chloroethyl)amino)phenylbutylamino- α -threoside nitrone (70)

Using aglycon **65** (35 mg, 0.11 mmol), the neoglycoside nitrone was yielded as a colorless syrup (23 mg, 49%, $R_f = 0.11$ MeOH:CH₂Cl₂ 5:95). ¹H NMR (CD₃OD, 500 MHz) δ 7.22 (d, $J = 5.8$ Hz, 1 H, H1'), 7.10 (d, $J = 8.6$ Hz, 2 H), 6.70 (d, $J = 8.6$ Hz, 2 H), 4.84 (dd, $J = 5.8, 4.2$ Hz, 1 H, H2'), 3.87–3.84 (m, 3 H, H3' + H4'), 3.75–3.72 (m, 4 H), 3.68–3.61 (m, 6 H), 2.59 (t, $J = 7.4$ Hz, 2 H), 1.93–1.87 (m, 2 H), 1.69–1.63 (m, 2 H); ¹³C NMR (CD₃OD, 125 MHz) δ 146.11, 146.01 (C1'), 132.10, 130.72, 113.71, 73.93 (C3'), 68.77 (C2'), 65.95 (C4'), 64.09, 54.70, 41.85, 35.30, 29.41, 27.93; HRMS (MALDI) m/z for C₁₈H₂₈Cl₂N₂NaO₄ ([M+Na]⁺) 429.13188, calc. 429.13183.

4-(4-*N'*,*N'*-bis(2-chloroethyl)amino)phenylbutylhydrazido- β -fucoside (72)

Using aglycon **71** (47 mg, 0.15 mmol), the product was yielded as a colorless syrup (46 mg, 67%, $R_f = 0.28$ MeOH:CH₂Cl₂ 10:90). ¹H NMR (CD₃OD, 500 MHz) δ 7.09 (d, $J = 8.6$ Hz, 2 H), 6.70 (d, $J = 8.6$ Hz, 2 H), 3.86 (d, $J = 8.7$ Hz, 1 H, H1'), 3.74–3.72 (m, 4 H), 3.68–3.65 (m, 4 H), 3.64–3.62 (m, 2 H, H3' + H5'), 3.54 (dd, $J = 9.5, 3.2$ Hz, 1 H, H4'), 3.48 (d, $J = 8.7$ Hz, 1 H, H2'), 2.56 (t, $J = 7.5$ Hz, 2 H), 2.21 (t, $J = 7.5$ Hz, 2 H), 1.90 (qui, $J = 7.5$ Hz, 2 H), 1.28 (d, $J = 6.4$ Hz 3 H); ¹³C NMR (CD₃OD, 125 MHz) δ 174.72, 144.86, 130.58, 129.53, 112.44, 91.30 (C1'), 74.29 (C4'), 72.12 (C3'), 72.03 (C5'), 68.38 (C2'), 53.45, 40.61, 34.02, 33.28, 27.65, 24.17, 15.93 (C6'); HRMS (MALDI) m/z for C₂₀H₃₁Cl₂N₃NaO₅ ([M+Na]⁺) 486.15233, calc. 486.15330.

4-(4-*N'*,*N'*-bis(2-chloroethyl)amino)phenylbutylhydrazido-D-glucurono-6,3-lactonide (73)

Using aglycon **71** (61 mg, 0.19 mmol), the mixture of compounds was yielded as a colorless syrup (42 mg, 44%, $R_{fA} = 0.55$ $R_{fB} = 0.47$ MeOH:CH₂Cl₂ 10:90; α : β :imine = 0:1:1). ¹H NMR (CD₃OD, 500 MHz) δ 7.57 (d, $J = 4.3$ Hz, 0.35 H, imine A-H1'), 7.41 (d, $J = 4.1$ Hz, 0.15 H, imine B-H1'), 7.09 (d, $J = 8.6$ Hz, 2 H), 6.70 (d, $J = 8.6$ Hz, 2 H), 4.91 (dd, $J = 6.4, 4.7$ Hz, 0.5 H, β -H4'), 4.85–4.79 (m, 0.5 H, β -H3'), 4.78 (d, $J = 1.4$ Hz, 0.5 H, β -H1'), 4.68 (dd, $J = 7.6, 4.3$ Hz, 0.35 H, imine A-H2'), 4.63 (dd, $J = 7.8, 4.1$ Hz, 0.15 H, imine B-H2'), 4.57–4.54 (m, 1 H, imine-H4'/H5' + β -H2'), 4.53–4.50 (m, 1.35 H, imine A-H3' + imine-H4'/H5' + β -H5'), 4.46–4.44 (m, 0.15 H, imine B-H3'), 3.75–3.71 (m, 4 H), 3.68–3.65 (m, 4 H), 2.71–2.54 (m, 2 H), 2.27 (t, $J = 7.5$ Hz, 1 H), 2.20–2.15 (m, 1 H), 1.96–1.87 (m, 2 H); ¹³C NMR (CD₃OD, 125 MHz) δ 176.71 (imine-C6'), 176.08 (β -C6'), 171.83, 148.17 (imine A-C1'), 144.93, 144.85 (imine B-C1'), 130.37, 129.50, 112.43, 99.79 (β -C1'), 84.14 (β -C3'), 81.62 (imine B-C3'), 81.53 (imine A-C3'), 77.83 (β -C4'), 70.96 (imine B-C4'/C5'), 70.88 (β -C2'), 70.11 (imine AC-4'/C5'), 69.97 (imine B-C4'/C5'), 69.84 (imine A-C4'/C5'), 69.49 (β -C5'), 69.20 (imine A-C2'), 69.59 (imine B-C2'), 53.40, 40.62, 33.99, 33.58 (β -C2), 33.47 (imine-C2), 27.36; HRMS (MALDI) m/z for C₂₀H₂₇Cl₂N₃NaO₆ ([M+Na]⁺) 498.11631, calc. 498.11691.

4-(4-*N'*,*N'*-bis(2-chloroethyl)amino)phenylbutylhydrazido- α -threoside imine (74)

Using aglycon **71** (36 mg, 0.11 mmol), the neoglycoside imine was yielded as a colorless syrup (41 mg, 85%, $R_f = 0.19$ MeOH:CH₂Cl₂ 10:90). ¹H NMR (CD₃OD, 500 MHz) δ 7.53 (d, $J = 4.9$ Hz, 0.6 H, imine A-H1'), 7.37 (d, $J = 5.5$ Hz, 0.4 H, imine B-H1'), 7.08 (d, $J = 8.6$ Hz, 2 H), 6.70 (d, $J = 8.6$ Hz, 2 H), 4.32 (t, $J = 4.4$ Hz, 0.6 H, imine A-H2'), 4.25 (m, 0.4 H, imine B-H2'), 3.78–3.70 (m, 5 H, imine-H3'), 3.69–3.60 (m, 6 H, imine-H4'), 2.65 (t, $J = 7.5$ Hz, 0.8 H, imine B-H2), 2.58 (t, $J = 7.5$ Hz, 1.2 H, imine A-H2), 2.26 (t, $J = 7.5$ Hz, 1.2 H, imine A-H4), 1.98–1.90 (m, 2.8 H, imine-H3 + imine B-H3); ¹³C NMR (CD₃OD, 125 MHz) δ 171.67, 151.04 (imine A-C1'), 147.62 (imine B-C1'), 144.92, 130.40, 129.49, 112.44, 73.65 (imine B-C3'), 73.46 (imine A-C3'), 71.49 (imine B-C2'), 71.40 (imine A-C2'), 62.60 (imine-C4'), 53.42, 40.59, 34.19 (imine B-C2), 34.00 (imine A-C2), 33.56 (imine A-C4),

31.76 (imine B-C4), 27.41 (imine A-C3), 26.82 (imine B-C3); HRMS (MALDI) m/z for $C_{18}H_{27}Cl_2N_3NaO_4$ ($[M+Na]^+$) 442.12721, calc. 442.12708.

4-(4-N', N'-bis(2-chloroethyl)amino)phenylbutylhydrazido-D-xyloside (75)

Using aglycon **71** (60 mg, 0.19 mmol), the mixture of compounds was yielded as a colorless syrup (40 mg, 45%, $R_{fA} = 0.36$ $R_{fB} = 0.29$ MeOH:CH₂Cl₂ 10:90; α : β :imine = 0:2.3:1). ¹H NMR (CD₃OD, 500 MHz) δ 7.54 (d, $J = 5.0$ Hz, 0.15 H, imine A-H1'), 7.38 (d, $J = 5.5$ Hz, 0.15 H, imine B-H1'), 7.09 (d, $J = 8.6$ Hz, 2 H), 6.70 (d, $J = 8.6$ Hz, 2 H), 4.45 (d, $J = 3.8$ Hz, 0.15 H, imine A-H2'), 4.40 (t, $J = 4.9$ Hz, 0.15 H, imine B-H2'), 3.89 (dd, $J = 11.3$, 5.4 Hz, 0.7 H, β -H5_A'), 3.85 (d, $J = 8.7$ Hz, 0.7 H, β -H1'), 3.75–3.72 (m, 4.3 H, imine B-H3' + imine A/B-H4'), 3.68–3.65 (m, 4.75 H, imine A/B-H4' + imine-H5'), 3.59 (dd, $J = 7.8$, 4.0 Hz, 0.15 H, imine A-H3'), 3.52–3.45 (m, 0.7 H, β -H4'), 3.39–3.36 (m, 0.7 H, β -H3'), 3.23–3.16 (m, 1.4 H, β -H2' + β -H5_B'), 2.56 (t, $J = 7.5$ Hz, 2 H), 2.27–2.24 (m, 0.6 H, imine-H2), 2.21–2.17 (m, 1.4 H, β -H2), 1.96–1.87 (m, 2 H); ¹³C NMR (CD₃OD, 125 MHz) δ 174.67, 144.87, 130.53, 129.49, 112.43, 91.78 (β -C1'), 87.51 (imine A-C2'), 77.17 (β -C3'), 72.44 (imine A/B-C4'), 72.43 (imine B-C3'), 71.57 (imine A/B-C4'), 72.00 (imine B-C2'), 71.34 (β -C2'), 70.92 (imine A-C3'), 70.05 (β -C4'), 67.28 (β -C5'), 62.95 (imine-C5'), 53.43, 40.60, 33.99, 33.22, 27.67; HRMS (MALDI) m/z for $C_{19}H_{29}Cl_2N_3NaO_5$ ($[M+Na]^+$) 472.13712, calc. 472.13765.

Cell proliferation assays

Testing was performed by the Keck-UWCCC Small Molecule Screening Facility (Madison, WI). General carcinoma cell line maintenance, compound handling and assay protocols have been previously reported.²⁰ Briefly, cells were plated in 50 μ L volumes at a density of 500 cells per well in 384-well clear bottom tissue culture plates. Serial dilutions of 30 mM DMSO compound stock solutions were accomplished in 96-well plates using a BioTek Precision XS liquid handler (Winooski, VT) to a concentration 100 $\times$ greater than that of the most dilute assay. Final dilutions were performed in a 384-well plate in quadruplicate using a Beckman-Coulter Biomek FX liquid handler with a 384 channel pipetting head (Fullerton, CA) and were stored at -20 °C when not in use. Compounds were then added to the culture plates by the Biomek FX handler and were incubated at 37 °C for 7 d in an atmosphere containing 5% CO₂. The calcein AM reagent (acetoxymethyl ester; 10 μ M) and ETHD-1 (100 μ M; 30 μ L total) were added, the treated cells incubated for 30 m at 37 °C, and plates read for fluorescent emission using a Tecan Safire2 microplate reader (Duram, NC) at the appropriate wavelengths. Cell titer-glo reagent (15 μ L; Promega Corp., Madison, WI) was added and the plates incubated for 10 m at room temperature with gentle agitation to lyse the cells. Each plate was re-examined for luminescence to verify inhibition. GI₅₀ values for cytotoxicity were determined using XLfit 4.2 as previously reported.²⁰ For compound **60**, the most active neoglycoside, the NCI60 human tumor cancer cell line screen was performed by the Developmental Therapeutics Program of the National Cancer Institute (Rockville, MD) as previously described.^{61,62}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by funding from the NIH (U19 CA113297 and AI52218) and the Laura and Edward Kremer Chair in Natural Products Chemistry (J.S.T.). Analytical support was provided by the School of Pharmacy Analytical Instrumentation Center and cancer cell line cytotoxicity screening was provided by the Keck UWCCC Small Molecule Screening Facility.

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Figure 1. Chlorambucil (**1**) and other nitrogen mustard compounds including *N*-oxide **2** (PX-478), FDA-approved bendamustine (**3**), and glycosylated variants glufosfamide (**4**) and **5**. The latter was found to inhibit the brain/erythrocyte α -glucose GLUT1 transporter and thereby decrease glucose uptake (reference 42).

Figure 3. Structures of the most antiproliferative chlorambucil *N*-alkoxyamino-based neoglycosides against a ten-member carcinoma panel.

Figure 4. ^1H NMR spectra of *N*-hydroxyaminochlorambucil glycosides. **A.** Equilibrium between the cyclic neoglycoside and acyclic nitron of D -fucoside **66**. **B.** Nitron form of the chlorambucil D -threoside **70**. Both spectra obtained at 500 MHz in CD_3OD .

Figure 5.

Interconversion of *N*-hydroxyaminochlorambucil-*D*-ribose between the cyclic neoglycoside and acyclic nitron **68** and peracetylated analog **69**. Both spectra obtained at 500 MHz with **68** in CD_3OD and **69** in CDCl_3 .

Figure 6. ^1H NMR spectra of *N*-hydrazidochlorambucil glycosides. **A.** Equilibrium between the cyclic neoglycoside and acyclic imine of D -glucurono-6,3-lactonide **73**. **B.** Nitron form of the chlorambucil D -threoside **74**. Both spectra obtained at 500 MHz in CD_3OD .

Figure 7.

Summary of GI_{50} data from the high-throughput growth inhibition assay of **66–70** hydroxyamines and **72–75** hydrazides (reciprocal values displayed). Comparisons were performed against methoxyamines **39** and **60**, aglycons **65** and **71**, and chlorambucil (**1**). GI_{50} data and error values are provided in the Supporting Information, Table 5. Representative cancer cell lines tested include: NCI-H460 (lung), A549 (lung), Du145 (prostate), SKOV3 (ovary), Hep3b (liver), SF268 (brain), MCF7 (breast), HT29 (colorectal), HCT15 (colorectal), H1299 (lung).

Scheme 1.

Synthesis of the *N*-alkoxyamino-based chlorambucil library^a

^a(a) MeON(H)Me, NMM, EDAC, 0 °C (97%); (b) LAH, THF, 0 °C (70%); (c) MeONH₃Cl, Et₃N, EtOH (84%); (d) BH₃·Et₃N, HCl, EtOH, 0 °C (65%); (e) reducing sugar, MeOH, HOAc (1.5 eq.), 40 °C (63% avg.).

Scheme 2.

Synthesis of the *N*-acyl hydrazine- and *N*-hydroxyamino-based chlorambucil libraries^a

^a(a) HONH₃Cl, Et₃N, EtOH (98%); (b) BH₃·Et₃N, HCl, EtOH, 0 °C (65%); (c) reducing sugar, MeOH, HOAc (1.5 eq.), 40 °C (47% avg.); (d) (i) *N*-hydroxysuccinimide, DIC, THF, 40 °C, (ii) NH₂NH₂, pyridine, DMAP, 40 °C (85%); (e) reducing sugar, MeOH, HOAc (1.5 eq.), 40 °C (62% avg.).