



Synthesis of acylated glycoconjugates as templates to investigate in vitro biopharmaceutical properties

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ARTICLE INFO

Article history:

Received 11 October 2012

Revised 9 November 2012

Accepted 14 November 2012

Available online 29 November 2012

Keywords:

Carbohydrate

Prodrug

Esterase

Metabolic stability

Click chemistry

Azide

ABSTRACT

A series of novel glycopyranosyl azides were synthesised wherein the carbohydrate moiety was peracylated with four acetyl, propionyl, butanoyl, pentanoyl (valeryl) or 3-methylbutanoyl (isovaleryl) ester linked groups. A panel of glycoconjugates was synthesised from these glycopyranosyl azides using copper-catalysed azide–alkyne cycloaddition. The in vitro metabolic stability, plasma stability and plasma protein binding was then measured to establish the impact of the different acyl group when presented on a common scaffold. The acetyl, propionyl and butanoyl esters exhibited metabolism consistent with esterase processing, and various mono-, di- and tri-acylated hydrolysis products as well as the fully hydrolysed compound were detected. In contrast, the pentanoyl and 3-methylbutanoyl esters were stable.

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Introduction

Carbohydrates are polyhydroxylated compounds that when incorporated onto a small molecule may be used as a platform from which to modulate physicochemical and biopharmaceutical properties of the resulting compounds for medicinal chemistry applications.^{1–4} Carbohydrates linked to natural product based drugs are often a prerequisite for biological activity and heavily influence the pharmacokinetics, drug targeting and mechanism of action.⁵ The development of synthetic carbohydrate-based small molecules is increasingly being recognized as a viable and appealing approach towards safe and effective therapeutics^{1–4,6} and ester prodrugs of monosaccharides have been developed.^{7–9} Esters are the most common prodrug moiety employed by the pharmaceutical industry.¹⁰ They are used extensively to mask polar hydroxyl groups with hydrophobic acyl groups to enhance passive diffusion and enable therapeutic concentrations of the prodrug to reach the bloodstream. The ester functional group is susceptible to in vivo hydrolysis by hydrolytic plasma enzymes and cellular carboxylesterase (CE) enzymes to generate the corresponding alcohol and car-

boxylate.^{11,12} Human CEs play a key role in fatty acid metabolism, they comprise a number of isozymes with differential distribution, expression profile and ester substrate specificity and they differ to plasma esterase enzymes.¹⁰ The heterogeneity of substrate specificity of ester hydrolysis enzymes means that it is difficult to predict activity on differing acyl groups on parent alcohol drugs and this limits rational drug optimization.¹³ Yarema and colleagues investigated the bioactivity of *N*-acetyl-*D*-mannosamine (ManNAc) and butyrate and showed that in isolation each component had poor drug-like properties however when combined into a single carbohydrate molecule (Bu₄ManNAc) exhibited favourable biopharmaceutical properties.^{14,15}

In this Letter we describe the synthesis of novel glycoconjugates wherein the carbohydrate moiety is peracylated with acyl groups comprising acetyl, propionyl, butanoyl, pentanoyl (valeryl) or 3-methylbutanoyl (isovaleryl) groups. The in vitro metabolic stability, plasma stability and plasma protein binding were measured for one set of the glycoconjugates to establish the impact of the different acyl groups on these biopharmaceutical properties. To the best of our knowledge there has been no systematic approach in the medicinal chemistry of glycoconjugate small molecules to address the effect of labile acyl groups on biopharmaceutical properties associated with carbohydrate ester hydrolysis. As a consequence we were keen to provide new chemical tools in this context.

Abbreviations: CuAAC, copper-catalysed azide–alkyne cycloaddition; CE, carboxylesterase; clog_P, calculated Log_P; HLMS, human liver microsomes; HSA, human serum albumin; CYP450, cytochrome P450.

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Copper-catalysed azide–alkyne cycloaddition (CuAAC) with carbohydrate-based azide substrates is now routinely applied in drug discovery and has proven extremely versatile both in the preparation of glycoconjugate small molecule inhibitors against a range of enzymes and to append carbohydrates to biomolecules.^{16–18} Several reactions fulfill the criteria that defines click chemistry,¹⁹ however it is CuAAC to regioselectively form 1,2,3-triazoles that has proven the most accomplished click chemistry reaction for the development of new molecules with useful chemical properties, delivering an impressive volume of diverse applications within a short period.²⁰ In drug development some functional groups, while synthetically attractive, can be a liability to biopharmaceutical performance of a compound in vivo. The early applications of glycopyranosyl azide building blocks in click chemistry demonstrated that the formed triazole was compatible with commonly employed carbohydrate protecting group approaches²¹ and that peracetylated glycopyranosyl triazoles were robust molecules.^{21,22} Glycopyranosyl azides are stable and are inert towards a wide range of reaction conditions and are readily synthesised anomerically pure.¹⁶ In addition a range of bioactive glycopyranosyl triazoles have been reported.^{4,6,17,22–25}

The azide precursors of the glycoconjugate target compounds of this study, compounds **1–10**, are O-peracetylated β -D-glucopyranosyl and β -D-galactopyranosyl azides containing acetyl (**1** and **6**), propionyl (**2** and **7**), butanoyl (**3** and **8**), pentanoyl (**4** and **9**) and 3-methylbutanoyl (**5** and **10**) moieties, Figure 1. Compounds **1** and **6** are well known and have been widely used,¹⁶ however the remaining acyl analogues are novel compounds. To synthesise compounds **1** and **6** we employed the Lewis acid catalysed synthesis directly from commercially available per-O-acetylated D-glucose and per-O-acetylated D-galactose using trimethylsilyl azide¹⁶ to stereoselectively introduce the azide functional group at the anomeric centre. We then considered two approaches for the synthesis of azides **2–5** and **7–10**. The first approach operated similarly to the synthesis of **1** and **6**, with the intention to directly introduce the azide at the anomeric centre of the corresponding per-O-acylated glucose and galactose derivative. The per-O-acylated precursors needed to be synthesised and acylation of unprotected D-glucose with an acid anhydride using typical monosaccharide acylation conditions was investigated. The reaction of D-glucose with propionic anhydride and butyric anhydride in either refluxing pyridine or using sodium propanoate or sodium butanoate, respectively, generated an anomeric mixture of the peracylated sugars **11** and **12**, Scheme 1.^{26–28} While the target β -anomer predominated (typical ratio β : α ~80:20) it could not be readily separated from the α -anomer by chromatography or crystallization. We continued with compounds **11** and **12** as anomeric mixtures and successfully converted the β -anomer to azides **2** and **3** (the α -anomer is

unreactive), Scheme 1, extensive chromatography was however required for purification. The second approach employed peracetylated β -glycopyranosyl azides **1** and **6** as indirect precursors for the remaining target azides **2–5** and **7–10**, Scheme 2. Deprotection of **1** and **6** using Zemplén conditions gave the β -glycopyranosyl azides **13** and **14**, respectively, in an almost quantitative yield.²⁹ Next acylation of **13** and **14** with the corresponding propionic, butyric, pentanoic or 3-methyl butyric acid anhydride in pyridine gave the target per-O-acylated β -glycopyranosyl azides **2–5** and **7–10** in good to high yields. This second approach was preferred to the first as it provided good yields from accessible starting materials and circumvented the purification difficulty caused by the formation and use of anomeric mixtures. The ¹H and ¹³C NMR spectra of azide compounds **2–5** and **7–10** were consistent with their expected structures.

With the panel of anomeric glycopyranosyl azides **1–10** in hand next the 1,4-disubstituted-1,2,3-triazole glycoconjugate target compounds **15–24** were synthesised from these azides and phenylacetylene by CuAAC, Scheme 2. The ¹H and ¹³C NMR spectra of **15–24** were consistent with their expected structures, the H-4 proton of the 1,4-disubstituted triazoles resonated at δ 8.95–8.82 ppm, while the triazole CH carbon resonated at δ 120.6–120.3 ppm. These chemical shifts are in agreement with those reported for other 1,4-disubstituted-1,2,3-triazoles.³⁰

Compound **25** is the fully deacylated glycoconjugate that would result following the complete hydrolysis of acyl groups of the parent compounds **15–19**. Compound **25** was prepared from **15** using Zemplén conditions and was used as the control compound for the biopharmaceutical property assays with the glucose series compounds **15–19** described next. Esterase hydrolysis of compounds **15–19** may take place sequentially, to form multiple tri-acylated, di-acylated and mono-acylated glycoconjugates as intermediates, Scheme 3. The characteristic molecular weights of the potential intermediates allow monitoring by mass spectrometry.

In order to assess the susceptibility of these compounds to ester hydrolysis within plasma, we determined the in vitro stability of compounds **25** and **15–19** in human plasma at 37 °C, Table 1. The pentanoyl (**18**) and 3-methylbutanoyl (**19**) esters, together with the corresponding fully deacylated compound **25** were stable in human plasma. In contrast, the shorter chain acetyl, propionyl and butanoyl analogues (**15**, **16** and **17**) degraded with estimated half lives of 55, 26, and 555 min, respectively, the order of stability is butanoyl > acetyl > propionyl. Multiple mono-, di- and tri-acylated hydrolysis products as well as the fully hydrolysed compound **25** were detected for each labile compound consistent with serial hydrolysis of the ester moieties.

Given the broad range of calculated LogP (cLogP) values for the acylated glycoconjugates (+2.25 to +8.60, Table 1) and the likely

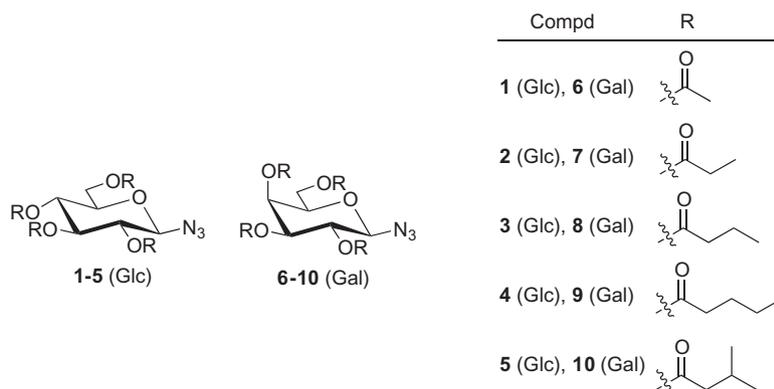
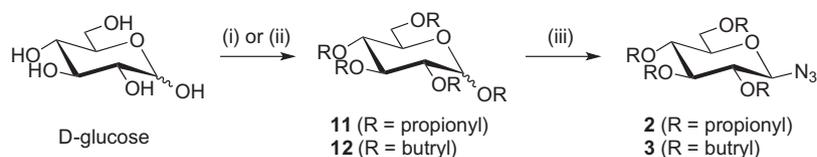
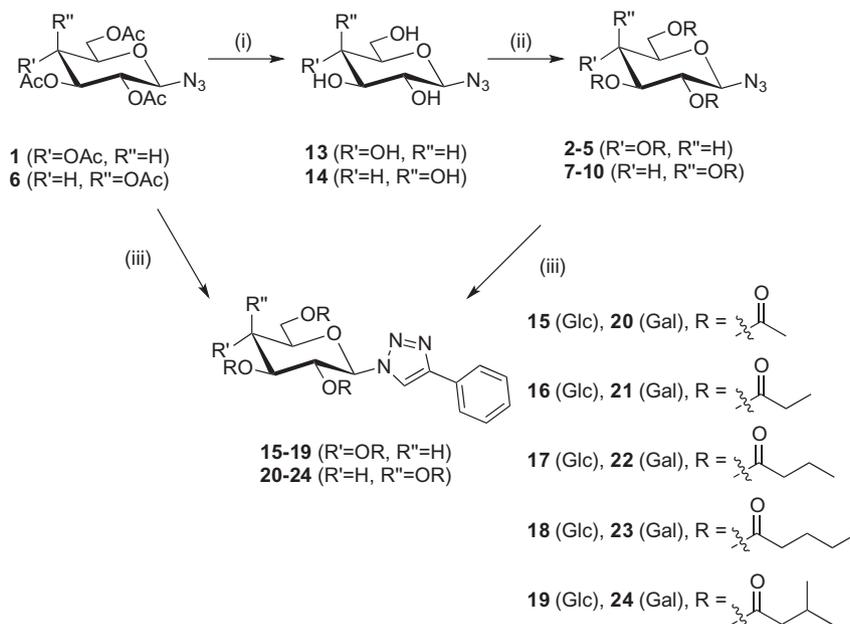


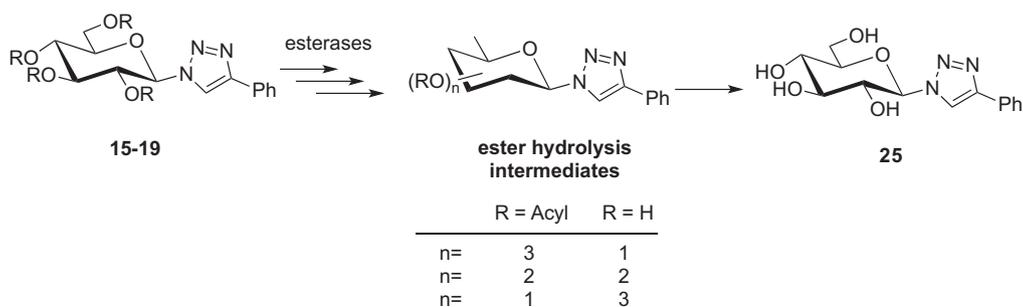
Figure 1. Target β -glucosyl azides **1–5** and β -galactosyl azides **6–10** each comprising four acetyl, propionyl, butanoyl, pentanoyl and 3-methylbutanoyl acyl groups.



Scheme 1. Approach to the synthesis of glycopyranosyl azides: synthetic approach 1. Reagents and conditions: (i) propionic anhydride for **11** or butyric anhydride for **12**, pyridine, reflux, 16 h to give β : α ~80:20, yield 80% (**12**); (ii) propionic anhydride and propionic acid sodium salt for **11** or butyric anhydride and butyric acid sodium salt for **12**, 150 °C, 30 min to give β : α ~70:30, yield 64% (**12**); (iii) SnCl₄ in CH₂Cl₂ (1.0 M, 1.6 equiv), TMS-N₃ (3.3 equiv), 0 °C to rt, 16 h, yield 90% (**3**).



Scheme 2. Synthesis of glycopyranosyl azides **2-5** and **7-10** (synthetic approach 2) and glycoconjugates **15-24**. Reagents and Conditions: (i) NaOMe in MeOH (25% w/v), MeOH, rt, 30 min–16 h, yield 94–95%; (ii) pyridine, acid anhydride (20 equiv), 60 °C, 16 h–2 d, yield 74–92%; (iii) CuAAC: azide 1–10 (1 equiv), phenylacetylene (10.0 equiv), CuSO₄·5H₂O (0.2 equiv), sodium ascorbate (0.4 equiv), H₂O:EtOH (1:2.5 to 1:5, 2.5–5 mL EtOH), 60 °C, 4 h–4 d, yield 60–86%.



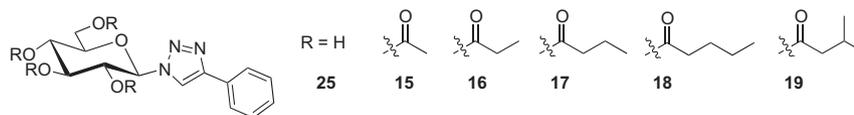
Scheme 3. Sequential hydrolysis of ester groups of glycoconjugates **15-19** to form the fully deacylated compound **25**.

relationship between lipophilicity and binding to serum albumin, we considered whether the differences in plasma stability of **15-19** could in part be due to differences in binding to plasma proteins. Experimentally, the lability of **15** and **16** precluded determination of protein binding via traditional methods (e.g., equilibrium dialysis, ultrafiltration and ultracentrifugation), due to the potential for deacylation under the experimental conditions. Instead, protein binding values of the test compounds were estimated by correlation of their chromatographic retention times on an immobilized human serum albumin (HSA) column against the characteristics of a series of standard compounds with known protein binding values.³¹ HSA binding increased with increasing size and lipophilicity of the acyl group and the two most highly bound

compounds (**18** and **19**) were also the most stable in human plasma, Table 1. On this basis it seems likely that the relative plasma stability of the different esters is determined by a combination of the intrinsic susceptibility to hydrolytic enzymes and the extent of binding to plasma proteins. Noncovalent plasma protein binding may be a desirable characteristic for diagnostic molecules such as MRI contrast agents, to increase the blood circulation half-life.³² In addition it has been demonstrated that plasma protein binding often has little clinical relevance on drug exposure.³³

We next determined the in vitro stability of the compounds **25** and **15-19** in human liver microsomes (HLMs) to provide a preliminary indication of the likely in vivo hepatic metabolic clearance and to see if metabolic products corresponding to loss of the acyl

Table 1
cLogP, in vitro human plasma stability, chromatographic HSA binding (cHSA) and in vitro HLM stability data for test compounds **15–19**, and **25**



Compd	Mwt	cLogP ^a	Plasma stability Degradation half-life ^b (min)	cHSAC (% bound)	HLM stability In vitro CLint (μL/min/mg protein) ^b	
					(+)NADPH	(-)NADPH
25	307.3	+0.11	n.d.d	19	<7	<7
15	475.5	+2.25	55	59	397	423
16	531.6	+4.37	26	88	c.n.c. ^e	c.n.c. ^e
17	587.7	+6.48	555	96	69	83
18	643.8	+8.60	n.d. ^d	<99 ^f	<7	<7
19	643.8	+8.08	n.d. ^d	<99 ^f	<7	<7

^c Values measured using the chromatographic retention times on an immobilized HSA column.

^a Calculated using ChemDraw Ultra 12.

^b Values are the average of duplicate incubations.

^d n.d. = no degradation.

^e c.n.c. = could not calculate, owing to rapid degradation.

^f Values are estimates only as the chromatographic peaks were broad and retention times could not be accurately determined.

groups, including complete loss (compound **25**) could be detected. Microsomal stability was assessed in both the presence and absence of NADPH, the cofactor required for oxidative activity of the cytochrome P450 (CYP450) enzyme family. This allowed a qualitative indication of the relative contributions of CYP450 and non-CYP450 mediated degradation processes. The pentanoyl (**18**) and 3-methylbutanoyl (**19**) compounds, together with compound **25**, were essentially stable in HLMs in both the presence and absence of NADPH; these compounds would therefore be expected to be subject to low hepatic metabolic clearance in vivo. In contrast, the acetyl, propionyl and butanoyl analogues (**15**, **16** and **17**) were degraded in HLMs in the presence of NADPH, and a comparable degradation rate for each compound in the absence of NADPH was evident, an indicator that microsomal metabolism was primarily due to non-CYP450 enzymes. Mono-, di- and tri-acylated hydrolysis products as well as the fully hydrolysed compound **25** were detected for each of the labile acyl derivatives, an indicator that the cofactor-independent degradation resulted from compound susceptibility to hydrolytic enzymes such as microsomal esterases.

LogP describes compound lipophilicity, and values often correlate with a number of key biopharmaceutical parameters in drug discovery. The use of software tools to calculate LogP (cLogP) is routine to provide rapid and accurate predictions when used with an awareness of limitations of the software.^{10,34} Table 1 contains calculated LogP (cLogP) values and molecular weight for glycoconjugates **15–19** and **25**. The trend of cLogP values is consistent with the compounds biopharmaceutical properties, discussed above. cLogP values increase with the size of the acyl group, it is feasible that the different esters could also be applied to balance oral absorption and bioavailability characteristics of prodrugs with esterase processing.

In summary, a synthetic route towards novel per-O-acylated glycopyranosyl azides with variable acyl groups was developed, this synthesis delivered anomerically pure tetra-acylated compounds in good yields. Using CuAAC the azide building blocks were then transformed into glycoconjugate templates and used as tools to study the effect of different acyl groups on biopharmaceutical properties. Specifically we determined the in vitro stability of variably acylated glycoconjugates **15–19** as well as the non-acylated control compound **25** in human plasma and in HLMs. Pentanoyl (**18**) and 3-methylbutanoyl (**19**) esters, together with the corresponding fully deacylated compound **25** were stable both in

human plasma and in HLMs, while the shorter chain acyl groups (acetyl, propionyl and butanoyl) of **15–17** exhibited degradation consistent with esterase processing, the order of stability was butanoyl > acetyl > propionyl. Various mono-, di- and tri-acylated hydrolysis products as well as the fully hydrolysed compound **25** were detected from metabolism of compounds **15–17**, consistent with serial hydrolysis of the carbohydrate ester moieties. The plasma protein binding to HSA was also measured and observed to increase with increasing size and lipophilicity of the acyl group. These findings may indicate that the relative plasma stability of the different esters is determined by a combination of the intrinsic susceptibility to hydrolytic enzymes and the extent of binding to plasma proteins. Collectively our findings demonstrate key differences in acyl group stability when presented on a common carbohydrate scaffold core. This may provide a useful guide to the selection of small acyl groups for regulating biopharmaceutical properties in future ester prodrug approaches.

Acknowledgment

This research was financed by the Australian Research Council (Grant number DP110100071 to S.-A.P. and S.A.C.) and Griffith University (Student Scholarship to C.J.C.).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2012.11.056>.

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