Technical Note

Trypanosoma cruzi ¹³C-labeled O-Glycan standards for mass spectrometry

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Abstract

Trypanosoma cruzi is a protozoan parasite that causes Chagas disease, a debilitating condition that affects over 10 million humans in the American continents. In addition to its traditional mode of human entry via the “kissing bug” in endemic areas, the infection can also be spread in non-endemic countries through blood transfusion, organ transplantation, eating food contaminated with the parasites, and from mother to fetus. Previous NMR-based studies established that the parasite expresses a variety of strain-specific and developmentally-regulated O-glycans that may contribute to virulence. In this report, we describe five synthetic O-glycan analytical standards and show their potential to enable a more facile analysis of native O-glycan isomers based on mass spectrometry.

Key words: mass spectrometry, Parasitology, *Trypanosoma cruzi*, O-glycans

Introduction

Trypanosoma cruzi is the cause of Chagas disease, the highest impact infectious disease of the Americas with 10–20 million humans affected (Tarleton 2016). Although *T. cruzi* infection is generally controlled by host immune responses, it is rarely cleared, resulting in a chronic infection, with the potential development of cardiac and/or gastrointestinal manifestations in approximately 30% of infected individuals. Chagas disease is plagued by poor diagnostics, inadequate treatment options and the absence of vaccines (Prata 2001).

Trypanosoma cruzi expresses a broad repertoire of protein and lipid-linked glycans implicated in differentiation, cell invasion, and regulation of the host immune response. Substantial glycosylation changes likely support success in the sequential environments confronted during its life cycle (Tyler and Engman 2001). O-linked glycans are particularly abundant on mucins and mucin-associated proteins expressed on the parasite surface. Two major groups of mucin-like GPI-anchored glycoproteins are inversely expressed on the proliferative insect dwelling stage and infective cell-derived

trypomastigotes (Buscaglia et al. 2006). The dense mucin arrays may protect epimastigotes from proteases in the insect intestine (Mortara et al. 1992), support survival of metacyclic trypomastigotes and their penetration of the gastric mucosal epithelium after ingestion by the mammalian host (Hoft et al. 1996), and protect bloodstream forms from complement attack (Tomlinson and Raper 1998). Mucins have also been implicated in T-cell suppression possibly through binding L-selectin (Argibay et al. 2002; Alcaide and Fresno 2004). Antibodies (Abs) against mucin-type O-glycans inhibit host-cell invasion (Yoshida et al. 1989; Ruiz et al. 1993). Terminal α 3 Gal-linkages of infective trypomastigotes induce Chagasic anti- α Gal Abs that can reduce parasite burden by a complement-independent mechanism (Gazzinelli et al. 1999), and may affect binding to host galectins (Pineda et al. 2014). Gal₆ is a novel and immunogenic sugar of many pathogens that is also found on *T. cruzi* O-glycans (Oppenheimer et al. 2011). Sialic acids are applied to O-glycans and other glycans by a family of novel transia-lidases (Freire-de-Lima et al. 2015) and have been implicated in reduced and increased infectivity, attachment to and invasion of

Mass spectrometry (MS) is an alternative method with the potential to comprehensively survey the O-glycome of small amounts of material, and MSⁿ approaches hyphenated with separation methods have great potential to characterize isomers with regard to anomericity, regiospecificity, and ring size and identity of the component sugars. However, chemically defined standards are needed to work out the methods. While certain *T. cruzi* O-glycans have been previously synthesized (Mendoza et al. 2010; Kashiwagi et al. 2012; Agustí et al. 2015; Schocker et al. 2016; Giorgi et al. 2017), the compounds are in limited supply and not generally available to the community. Here we characterize five chemo-enzymatically synthesized isomers that represent known *T. cruzi* mucin-type O-glycans and contain the main constituents known to be biosynthetically assembled in the parasite secretory pathway. It is anticipated that

To examine the utility of the compounds for the analysis of *T. cruzi* O-glycans, an extract from epimastigotes was subjected to standard conditions of reductive β -elimination, permethylated, and chromatographed in parallel. All glycans that eluted prior to 90 min (separation range for the synthetic standards) could be assigned as

ID	Composition	SNFG/Oxford representation	Molecular formula	Molecular mass, Da (monoisotopic)	¹³ C-Atom enrichment	Chemical Purity ^a		
						HPLC	¹³ C-NMR	¹ H-NMR
TRI-023	Galpα1,3[U- ¹³ C ₆]Galpβ1,4GlcNAcitol		¹³ C ₆ C ₁₄ H ₃₇ NO ₁₆	553.46	98%	95%	a	b
TET-023	Galpβ1,2Galpβ1,6([U- ¹³ C ₆]Galpβ1,4)GlcNAcitol		¹³ C ₆ C ₂₀ H ₄₇ NO ₂₁	715.60	99%	98%	a	b
TET-024	Galpβ1,2Galpβ1,6([U- ¹³ C ₆]Galpβ1,3)GlcNAcitol		¹³ C ₆ C ₂₀ H ₄₇ NO ₂₁	715.60	99%	>99%	a	b
TET-025	Galpβ1,2Galpβ1,6([U- ¹³ C ₆]Galpβ1,4)GlcNAcitol		¹³ C ₆ C ₂₀ H ₄₇ NO ₂₁	715.60	99%	>99%	a	a
TET-026	Galpβ1,3Galpβ1,6([U- ¹³ C ₆]Galpβ1,4)GlcNAcitol		¹³ C ₆ C ₂₀ H ₄₇ NO ₂₁	715.60	99%	>99%	a	a

^aProvided by the supplier: a = identity confirmed, no extraneous peaks detected; b = identify confirmed, no significant extraneous peaks detected.

Fig. 1. nLC-MS analysis of standard and *T. cruzi* O-glycans. **(A)** O-glycans were released from a preparation of *T. cruzi* mucins by reductive β -elimination, permethylated, and subject to nLC-MS on standard proteomics workstation utilizing a C18 column and a Thermo Velos Pro ITMS. Shown is the base-peak chromatogram of ions (500–2000 m/z) eluting from 12–98 min, with composition assignments based on exact monoisotopic mass ($[M + \text{Li}]^{+1}$ or $[M + 2\text{Li}]^{+2}$). H, hexose; N, HexNAc; Sa, NeuNAc or sialic acid. **(B)** An equimolar mixture of the five standards were chromatographed in parallel. The structures are illustrated using a blend of the CFG and Oxford symbols, to highlight the linkage differences. **(C)** Extracted ion chromatogram of mono-lithiated ions from the data in panel A zooming from 20 to 70 min to show glycans with a composition of H2N1. All compositions were confirmed by MS², which differentiated linear from branched trisaccharides, as indicated. **(D)** Similar analysis to show glycans with an H3N1 composition. All compositions were confirmed by MS² (except for RT 57.97). **(E)** Similar analysis of the data in panel B for the standards. Vertical red dashed lines highlight positions of co-elution with the standards.

ranging from a neutral disaccharide to an anionic tetrasaccharide and a neutral pentasaccharide, based on m/z -based composition analysis (annotated in Fig. 1A and summarized in Supplementary data, Table I). Other minor glycan species that eluted after 90 min included additional tetramer isomers, pentasaccharides and hexasaccharides (Supplementary data, Table I). Zooming in on the trisaccharide region revealed six isomers with a composition of H2N1 (Fig. 1C), none of which co-eluted with the trisaccharide standard

(Fig. 1E), as expected because this structure is expressed at a different stage of the life cycle. MS² (not shown) indicated that two of the isomers were linear while the other four were branched. Zooming in on the tetrasaccharide region revealed eight isomers with a composition of H3N1, and each was branched based on diagnostic ions in MS² (not shown). Only the isomer eluting at 43.6 min is a potential match to a known standard, which has been described in the CL-Brener strain (Mendonça-Prevato et al. 2013).

To investigate their potential to yield fragment ions for the analysis of unknown glycans, the standards were directly infused into a hybrid Ion Trap-Orbitrap mass spectrometer (Orbitrap Elite) using nanospray ionization in positive ion mode in the presence of LiOAc. The standards containing a β 4-linked lower arm Gal yielded a unique cross-ring fragment ion in MS² that was not observed in the standard containing a β 3-linked lower arm Gal (Supplementary data, Fig. 2A, C), whereas the latter yielded a characteristic Z-ion product in MS²⁻⁴ experiments (Supplementary data, Fig. 2B). The standard containing a β Gal 2-linked to the upper arm β 6-linked Gal was differentiated by a unique fragment ion (m/z 95) in MS⁵ that was not observed in the isomer containing a non-reducing terminal β 3Gal (Supplementary data, Fig. 2F), which was characterized by greater neutral losses of water and higher abundance of certain cross-ring cleavage products. Isomers that vary only in the pyranose or furanose configuration of the lower arm β 3Gal were more difficult to differentiate by this method, though a characteristic fragment ion (m/z 128) was relatively more abundant for the latter isomer at the MS⁵ level (Supplementary data, Fig. 2G).

To further investigate the identity of the *T. cruzi* H3N1 isomer co-eluting at 43.6 min with Gal β 1,2-Gal β 1,6-[Gal β 1,4]-GlcNAcitol (TET-023), we analyzed a mixture of the *T. cruzi* sample and TET-023. As shown in Supplementary data 2, Fig. 3, the ¹³C-isomers co-elute precisely with the minor contaminating ¹²C-isomers present in the samples, indicating the utility of the ¹³C-isomer as an internal standard. MSⁿ analysis of the *T. cruzi* H3N1 (¹²C-labeled) ion in the mixture up to MS⁵ yielded fragment ions that were indistinguishable from that of the standard, indicating that it is identical to TET-023 (Supplementary data, Fig. 4).

These data establish the value for the glycan standards to differentiate, at the level of HPLC retention time and MSⁿ, many different isomers of *T. cruzi* O-glycans. They are expected to be highly useful for optimizing separation and assignment platforms not only for *T. cruzi* but for O-glycans from all species containing related isomers. The standards have been made possible by an NIH Common Fund grant, and aliquots are freely available to the scientific community. Additional information and requests for standards can be obtained at the website (<https://ast.uga.edu/t-cruzi-o-glycans/>) or by directly contacting the corresponding author.

Materials and methods

See the Supplementary data.

Supplementary data

Supplementary data is available at GLYCOBIOLOGY online.

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Conflict of interest statement

None declared.

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