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A one-step chemical derivatization strategy for mass spectrometric characterization of synthetic mimetics of sulfated glycosaminoglycans

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We present a novel one-step methodology for direct mass spectrometric characterization of sulfated non-saccharide glycosaminoglycan mimetics in their sodiated form using the high reactivity of trifluorodiazoethane to form hydrophobic and chemically stable esters that facilitates separation and detection in a liquid chromatography–mass spectrometry system. The methodology preserves all sulfates present in the parent molecule, thereby allowing for accurate mass characterization of the per-sulfated molecule.

Glycosaminoglycans (GAGs) are highly heterogeneous and polydisperse natural products that are composed of variably sulfated, repeating disaccharide units. GAGs are ubiquitous in mammalian tissues and play essential roles in a variety of biological functions, including cell signaling, blood coagulation, and maintenance of the extracellular matrix.[1] Owing to their diverse functions, specific GAG-like sequences possess high therapeutic value.[2–4] Unfortunately, the highly sulfated structure of these sequences presents major challenges for synthesis and structural characterization,[5,6] two critical components in the development of small molecule drugs.

To address these challenges, non-saccharide GAG mimetics (NSGMs) have been developed. Whereas GAGs have a glycan scaffold, NSGMs are based on an aromatic scaffold, which affords synthetic ease and homogeneity.[7] The mimicking capability of NSGMs arise from the presence of multiple sulfate groups on an aromatic scaffold, which induces binding to GAG-binding domains present on proteins with concomitant GAG-like biological effects. The NSGM technology has now afforded highly promising agents including anti-cancer stem cell (CSC), anti-thrombotic, and anti-inflammatory agents.[8–10] One anti-CSC agent, labelled **G2.2** (Fig. 1), is particularly promising and has been shown to be a structural and functional mimetic of heparan sulfate hexasaccharide (Fig. S1). **G2.2** has been shown to selectively inhibit CSCs and prevent cancer progression in small rodents with minimal cellular or organ toxicity.[11]

The high promise of distinct NSGMs necessitates detailed structural characterization in their development as putative drugs. Unfortunately, sulfate groups are highly labile under mass spectrometric conditions, which complicate accurate mass elucidation, especially of the per-sulfated species.[12,13] Several strategies have been explored to mitigate such sulfate losses including soft ionization techniques[14] and chemical derivatization of sulfate groups to more stable functional groups.[15]

In one approach, sharpe and co-workers converted sulfate groups to acetyl groups that are not lost under MS conditions and also improve chromatographic resolution. However, this method involves multiple low-yielding chemical steps, which limit its applicability.[16] Related strategies, such as conversion to alkyl or aryl sulfate esters, have also shown better ionization efficiency and chromatographic retention. For example, neopentyl, trichloroethyl or trifluoroethyl groups have been

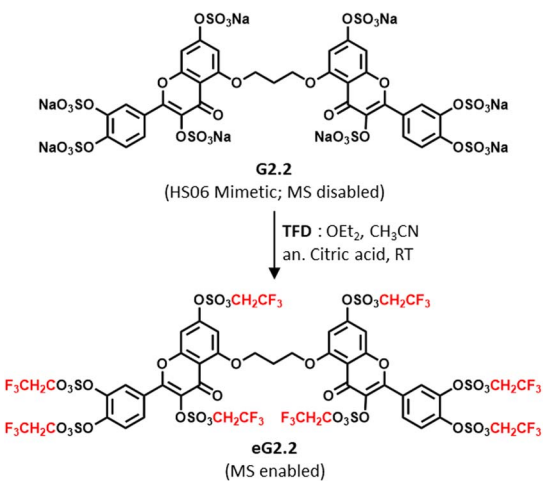

Fig. 1 One-step chemical derivatization of sulfated NSGM **G2.2** using 2,2,2-trifluorodiazoethane (TFD).

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introduced to improve volatility, hydrophobicity and stability of sulfonate esters.[17]

2,2,2-Trifluorodiazoethane (TFD) has been proven to be a valuable reagent for both academic and industrial research.[18] It was first discovered for sulfonic acid protection[19] and later implemented for protecting sulfate groups in carbohydrates that can be deprotected under basic conditions.[20] Linhardt *et al.* used TFD in the synthesis of sulfated building blocks of GAG.[21] Here, the triethylammonium salts of sulfated glucosamine and galactosamine were treated with excess TFD to yield trifluoroethylsulfate ester derivatives, which served as protected intermediates.

Interestingly, natural GAGs exist in nature in their sodiated form. Although mass spectrometry of sodium salts of natural GAGs is highly desirable, the application of TFD to enable MS of sulfated GAGs in their sodiated state is unknown. Here, we report the exploitation of the intrinsic reactivity of TFD to convert anionic sulfate groups of NSGMs to their alkylated form, which significantly enhances hydrophobicity, volatility and chemical stability, thereby enabling chromatographic separation as well as detection under mass spectrometric conditions. Our optimized protocol preserves each sulfate group of the precursor allowing for accurate mass determination. Our method offers a powerful tool for the structural characterization of sulfated NSGMs and will also enable high-resolution mass spectral characterization of natural sulfated GAGs.

G2.2 contains two quercetin moieties each carrying four sulfate groups at 3, 7, 3′ and 4′ positions (Fig. 1). It is synthesized as a sodiated salt, which exists in the solid state at room temperature. **G2.2** Remains stable for days in both solid and aqueous solution forms. In contrast, it rapidly loses one or more sulfate groups under typical electrospray ionization (ESI) MS conditions (Fig. S2).

To assess whether all sulfate groups of **G2.2** can be fully esterified, we first generated TFD in acetonitrile, as previously reported,[19,22] and introduced solid **G2.2** in the presence of anhydrous citric acid at room temperature. Following a 24-hr incubation, the LC profile of solution displayed multiple peaks (Fig. 2A). ESI-MS analysis revealed that most hydrophobic peak, eluting at 9.6 min, corresponded to the fully esterified **G2.2** molecule, as evidenced by characteristic doubly charged species $[M + 2Na]^{2+}$ at *m/z* 991.25 (Fig. 2B). Other peaks of lower hydrophobicities were found to correspond to partially esterified and/or desulfated species of **G2.2** (Table S1 and Fig. S3). The approximate yields of the species formed in the reaction, *i.e.*, peaks '*a*' to '*j*' (Fig. 2A), were calculated to be 0.5–5.0%. Alternatively, more than 85% of the starting material remained intact, yet detectable due to the exquisite sensitivity of MS.

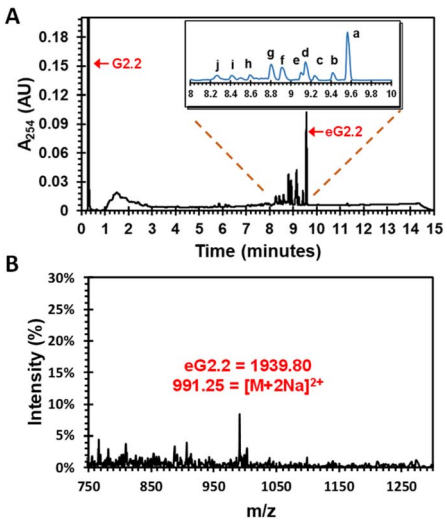


Fig. 2 (A) Chromatogram of TFD-treated **G2.2** with an inset showing zoomed in version. (B) ESI-MS spectrum of the doubly sodiated adduct of **eG2.2**, $[M + 2Na]^{2+}$.

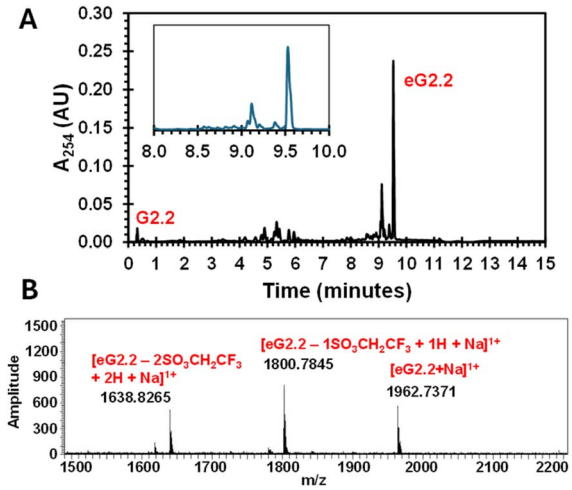
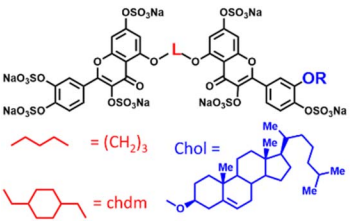


Fig. 3 (A) Chromatogram of TFD-treated **G2.2**. (B) HR-MS of a micro-scale, worked-up, extract of **G2.2**—TFD reaction showing the presence of **eG2.2** $[M + Na]^+$. Partially esterified species containing 6 and 7 CH_2CF_3 groups, suffering from *in situ* loss of two and one SO_3 groups, respectively, were also observed. See Fig. S2 for detailed ESI-MS detection of the range of partially esterified and desulfated species.

It is important to note that TFD is a highly reactive reagent that may explosively release N_2 under certain conditions. We did not experience any explosive incident in our work probably because we generated TFD *in situ* from commercially available chemicals under fairly dilute conditions.[18]

To assess whether the reaction can be driven in the direction of the per-esterified **eG2.2** only, we studied a range of factors including (i) the need for citric acid as a proton donor, (ii) the aqueous form of citric acid rather than its anhydrous solid form, (iii) alternative proton donors, *e.g.*, tetrafluoroboric acid (HBF_4), (iv) effect of temperature, and (iv) reactant loading capacity. Absence of citric acid yielded no esterified species



NSGM	L	R
G2.2	$(CH_2)_3$	SO_3Na
G2C	$(CH_2)_3$	$(CH_2)_2$-Chol
G5C	$(CH_2)_3$	$(CH_2)_5$-Chol
G8C	$(CH_2)_3$	$(CH_2)_8$-Chol
MQD	chdm	SO_3Na
MQD2C	chdm	$(CH_2)_2$-Chol
MQD5C	chdm	$(CH_2)_5$-Chol
MQD8C	chdm	$(CH_2)_8$-Chol

Fig. 4 Structures of library of NSGMs studied.

Table 1 HR-MS of NSGMs

Sl. no	Compound	Calculated mass (+adduct)	Experimental mass (error in ppm)
1	**eG2.2**	1962.7847 (+Na)	1962.7371 (24.3)
2	eG2C	2213.1953 (+Na)	2213.1658 (13.3)
3	eG5C	2233.2603 (+H)	2233.2895 (13.1)
4	eG8C	2275.3072 (+H)	2275.3127 (2.4)
5	eMQD1	2008.8653 (+H)	2008.8786 (6.6)
6	eMQD2C	2259.2759 (+H)	2259.2155 (26.7)
7	eMQD15C	2301.3229 (+H)	2301.3365 (5.9)
8	eMQD18C	2365.3518 (+Na)	2365.3424 (4.0)

confirming that a proton donor is essential. Likewise, aqueous citric acid resulted in no observable product, even after 24 hours, perhaps because water degrades electrophilic diazo intermediates.[23] We reasoned that HBF_4, a stronger acid, may serve as a more effective proton donor.[24] Indeed, partially esterified species were observed as early as 5 minutes into the reaction (not shown). However, only 5% of the product was per-esterified **eG2.2**; perhaps the acidity of HBF_4 was too high. We raised the reaction temperature to 37 °C and observed an increased conversion (14%) to **eG2.2**. However, the higher temperature also encouraged desulfation. In addition, some etherification of the desulfated phenols was observed.

Failure to identify better proton donors and incubation temperature led us to develop an operationally simple, mild, relatively short, and safe protocol with solid citric acid and ethereal TFD. The need for solid forms of both citric acid and **G2.2** suggested that the esterification occurs at solid–liquid interface (Fig. S4). In this heterogeneous environment, proton transfer from the citric acid to TFD, which is necessary for the generation of the reactive, transient diazonium intermediate, is likely to be conversion determining factor. To test this, we varied amounts of the two species aided by sufficient homogenization. Upon steadily increasing the **G2.2** amounts from 20 μg to 400 μg, we found a maximal conversion efficiency of 52% at 100 μg (Fig. 3A and S5). This implies mass transfer effects arising from a solid–liquid interfacial reaction. Upon a micro-scale workup (see Methods), the presence of **eG2.2** could be rigorously identified in the extract using high-resolution-mass spectrometry (HR-MS) (Fig. 3B), thereby confirming the presence of eight sulfate groups in **G2.2**.

The β-trifluoroethyl sulfate esterified **G2.2**, *i.e.*, **eG2.2**, displayed excellent chemical and thermal stability. In contrast to conventional sulfate esters bearing β-hydrogens, which are known to undergo elimination or hydrolysis, trifluoroethyl sulfate sulfates resist such degradation due to the absence of β-elimination pathways and the strong inductive effect of fluorine atoms.[25] This stabilization improves their detectability in LC-MS. Hence, the TFD-based protocol enabled detection and structural analysis of sulfate-rich NSGMs by LC-MS and HR-MS, whereby identifying the maximum number of sulfate groups present on scaffold is achieved with high fidelity.

To assess whether this protocol can be implemented for other NSGMs, we studied a group of lipid-conjugated sulfated small molecules found to be highly promising inhibitors of colorectal cancer (Fig. 4). The TFD-based one-step protocol could be easily implemented for each of these molecules confirming its wider applicability (Table 1 and Fig. S6–12).

This study has led to the development of a robust, reproducible and effective one-step TFD-based chemical derivatization strategy for identifying the maximal sulfation level of synthetic mimetic of GAGs. The use of TFD for sulfoesterification remarkably enhances the resolution and detectability of unique molecules by enhancing their hydrophobicity, volatility and stability. This method addresses a key structural challenge of highly sulfated small molecules, *i.e.*, how do we know how many sulfate groups are present on a scaffold considering their extreme hydrophilicity and lability in —a mass spectrometer? similar problems also impede detailed direct structural characterization of GAGs, especially at microgram scales. It is likely that this method will greatly aid developing a robust HR-MS approach for the sulfated GAGs, which present a higher challenge in terms of heterogeneity and polydispersity.

Author contributions

C. S. and D. K.A. performed all mass spectrometry experiments. BKV synthesized the sulfated molecules. C. S. prepared the initial draft of the manuscript. U. R. D. led hypothesis generation, experimental design and analysis, manuscript finalization, and funding acquisition.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information: methods for TFD generation and reaction with NSGMs and detailed MS characterization profiles of NSGMs. See DOI: https://doi.org/10.1039/d5ay01855b.

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Notes and references

1 C. L. R. Merry, U. Lindahl, J. Couchman and J. D. Esko, in *Essentials of Glycobiology*, ed. A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart, M. Aebi, D. Mohnen, T. Kinoshita, N. H. Packer, J. H. Prestegard, R. L. Schnaar and P. H. Seeberger, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 4th edn, ch. 17, 2022.
2 R. S. Boothello, N. V. Sankaranarayanan, J. C. Sistla, B. Nagarajan, C. Sharon, J. E. Chittum, R. Y. Niyaz, S. Roy, A. Nandi, C. P. O'Hara, R. N. Gangji, D. K. Afosah, R. Ongolu, B. B. Patel and U. R. Desai, *Angew. Chem., Int. Ed.*, 2022, **61**, e202211320.
3 M. Chhabra, J. C. Wilson, L. Wu, G. J. Davies, N. S. Gandhi and V. Ferro, *Chemistry*, 2022, **28**, e202104222.
4 K. Arnold, Y. Xu, E. M. Sparkenbaugh, M. Li, X. Han, X. Zhang, K. Xia, M. Piegore, F. Zhang, X. Zhang, M. Henderson, V. Pagadala, G. Su, L. Tan, P. W. Park, R. T. Stravitz, N. S. Key, R. J. Linhardt, R. Pawlinski, D. Xu and J. Liu, *Sci. Transl. Med.*, 2020, **12**, eaav8075.
5 S. Perez, O. Makshakova, J. Angulo, E. Bedini, A. Bisio, J. L. de Paz, E. Fadda, M. Guerrini, M. Hricovini, M. Hricovini, F. Lisacek, P. M. Nieto, K. Pagel, G. Paiardi, R. Richter, S. A. Samsonov, R. R. Vivès, D. Nikitovic and S. Ricard Blum, *JACS Au*, 2023, **3**, 628–656.
6 E. I. Abdelfadiel, R. Gunta, B. K. Villuri, D. K. Afosah, N. V. Sankaranarayanan and U. R. Desai, *J. Med. Chem.*, 2023, **66**, 4503–4531.
7 B. K. Villuri and U. R. Desai, *Chemistry*, 2024, **30**, e202402268.
8 N. J. Patel, R. Karuturi, R. A. Al-Horani, S. Baranwal, J. Patel, U. R. Desai and B. B. Patel, *ACS Chem. Biol.*, 2014, **9**, 1826–1833.
9 R. A. Al-Horani, D. Gailani and U. R. Desai, *Thromb. Res.*, 2015, **136**, 379–387.
10 D. K. Afosah, R. M. Fayyad, V. R. Puliafico, S. Merrell, E. K. Langmia, S. R. Diagne, R. A. Al-Horani and U. R. Desai, *Biomolecules*, 2023, **13**, 760.
11 R. S. Boothello, N. J. Patel, C. Sharon, E. I. Abdelfadiel, S. Morla, D. F. Brophy, H. R. Lippman, U. R. Desai and B. B. Patel, *Mol. Cancer Ther.*, 2018, **18**, 51–61.
12 S. Morla, R. Ongolu, C. O'Hara, R. Boothello, A. Vera, E. I. Abdelfadiel, R. Fayyad, D. K. Afosah, C. Sharon, L. Fernandez, S. A. Shah, B. B. Patel and U. R. Desai, *J. Med. Chem.*, 2023, **66**, 1321–1338.
13 G. P. Szekeres, K. Pagel and Z. Heiner, *Anal. Bioanal. Chem.*, 2021, **414**, 85–93.
14 T. N. Laremore, F. E. Leach III, K. Solakyildirim, I. J. Amster and R. J. Linhardt, *Methods Enzymol.*, 2010, **478**, 79–108.
15 Q. Liang and J. S. Sharp, *Methods Mol. Biol.*, 2022, **2303**, 163–172.
16 H. Liu, Q. Liang and J. S. Sharp, *J. Am. Soc. Mass Spectrom.*, 2020, **31**, 2061–2072.
17 S. C. Miller, *J. Org. Chem.*, 2010, **75**, 4632–4635.
18 P. K. Mykhailiuk, *Chem. Rev.*, 2020, **120**, 12718–12755.
19 H. Gilman and R. G. Jones, *J. Am. Chem. Soc.*, 1943, **65**, 1458–1460.
20 A. D. Proud, J. C. Prodger and S. L. Flitsch, *Tetrahedron Lett.*, 1997, **38**, 7243–7246.
21 N. A. Karst, T. F. Islam and R. J. Linhardt, *Org. Lett.*, 2003, **5**, 4839–4842.
22 M. A. J. Duncton and R. Singh, *Org. Lett.*, 2013, **15**, 4284–4287.
23 N. A. McGrath, K. A. Andersen, A. K. F. Davis, J. E. Lomax and R. T. Raines, *Chem. Sci.*, 2015, **6**, 752–755.
24 K. L. Roller and H. C. Dorn, *Anal. Chem.*, 1982, **54**, 529–533.
25 P. G. Blake and B. F. Shraydeh, *Int. J. Chem. Kinet.*, 1982, **14**, 739–747.