Supplementary Information (SI) for Analytical Methods.

A One-Step Chemical Derivatization Strategy for Mass Spectrometric Characterization of Synthetic Mimetics of Sulfated Glycosaminoglycans

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1. Materials and Methods

All chemical reagents and solvents were purchased from commercial suppliers and used without further purification. Sodium nitrite, 2,2,2-trifluoroethylamine hydrochloride, inhibitor-free diethyl ether (≥99.9%), tetrafluoroboric acid solution (48 wt.% in H_2O), and tetrafluoroboric acid-diethyl ether complex were obtained from Sigma-Aldrich. Anhydrous citric acid, OPTIMA® LC/MS grade water, and acetonitrile were sourced from Fisher Scientific.

All reactions were conducted behind a blast shield using glassware inspected prior to use to ensure the absence of cracks or visible scratches.

2. Instrumentation

A Thermo Scientific Savant SpeedVac SPD140DDA medium-capacity vacuum concentrator, compatible with strong acids, and organic solvents, was used to concentrate the reaction mixtures after chemical derivatization of G2.2.

Ultrahigh-Performance Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry (UPLC-ESI-MS) was performed using a Waters ACQUITY TQD ESI-MS spectrometer operated in positive-ion mode. Samples were dissolved in a 4:1 (v/v) mixture of acetonitrile and water and analyzed by LC-MS. Separation of Esterified sulfated species was achieved on a Waters BEH C18 reversed-phase column (1.7 µm particle size, 2.1 mm × 50 mm) at a flow rate of 0.5 mL/min. Solvent A consisted of water with 0.1% (v/v) trifluoroacetic acid (TFA), and solvent B was acetonitrile with 0.1% (v/v) TFA.

Analytical Parameters

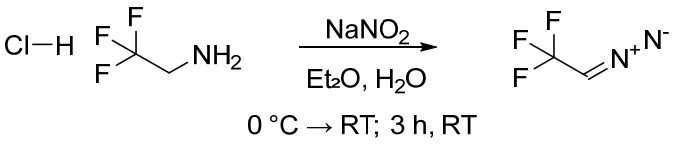
Column	BEH C18 reversed-phase column (1.7 µm particle size, 2.1 mm × 50 mm)	
Sample temperature	4°C	
Mobile phase A	0.1% TFA in Water	
Mobile phase B	0.1% TFA in Acetonitrile	
Flow rate	0.5 mL/min	
Gradient		
Time (min)	A%	B%
0	95	5
10	5	95
13	5	95
15	95	5

Mass spectrometric detection was carried out in positive-ion mode with capillary voltage of 4 kV, cone voltage of 20 V, desolvation temperature of 487 °C, and nitrogen gas flow at 650 L/h. Mass spectra were acquired over a range of 300-1900 amu with a scan time of 0.25 s. Multiple scans were averaged to improve the signal-to-noise ratio.

Instrument	ACQUITY UPLC with ACQUITY PDA
Ion Source Type	ESI
Mode	Positive-ion
Cone Voltage	20 V
Desolvation Temperature	487 ºC
Nitrogen gas flow	650 L/h
Mass scan range	300 - 1900 amu
Scan time	0.25 s

High-resolution mass spectrometry (HRMS) analyses were carried out using a PerkinElmer AXION 2 Time-of-Flight (TOF) mass spectrometer. The dried reaction mixtures were reconstituted in HPLC-grade acetonitrile. The reconstituted samples were then directly infused into the mass spectrometer under standard electrospray ionization (ESI) conditions. The instrument was operated in positive ion mode, with a flight voltage set to 8 kV, optimized for accurate mass detection and resolution.

3. Synthesis of TFD



Procedure: Sodium nitrite (50 mg, 0.725 mmol) in water (0.5 mL) was added in one portion to a stirred solution of 2,2,2- trifluoroethylamine hydrochloride (86 mg, 0.63 mmol) in water (1 ml) and ether (1.2 ml) at 0 °C. The reaction vessel was sealed with a teflon stopper and the mixture stirred from 0 °C to room temperature. Then it was allowed to stir at room temperature for approximately 3 hours. The ether layer containing the product was used directly in the next step without further purification. The yield of the trifluoromethyl diazomethane (TFD) product was determined to be ~ 50% by reacting the ethereal TFD solution with Iodine.

4. Chemical derivatization of NSGMs

General Procedure: **G2.2** (2 mg, 1.39 µmol) was suspended in acetonitrile (500 µL) and treated with 1 mL of a freshly prepared 0.3 M ethereal solution of 2,2,2-trifluorodiazoethane. Citric acid (150 mg) was then added, and the reaction mixture was stirred at room temperature. At 5 minutes, 16 hours and 24 hours, 50 µL aliquots of the reaction mixture were removed, dried using a speed vacuum concentrator, redissolved in 50 µL, 4:1 acetonitrile and water, and injected into UPLC-MS for analysis.

The influence of acid donors was investigated by conducting the reaction under different conditions: without citric acid, with the addition of 20 µL of ethereal HBF_4, and with 20 µL of aqueous HBF_4. Aliquots were collected over time and analyzed as described above. Additionally, the reaction was performed using aqueous **G2.2** instead of solid substrate to assess its impact on derivatization efficiency.

The chemical derivatization of other NSGMs (**G2C**, **G5C**, **G8C**, **MQD1**, **MQD2C**, **MQD15C** and **MQD18C**) were also performed using similar procedure from freshly prepared TFD. Following a micro-workup, the crude reaction mixture was subjected to HRMS.

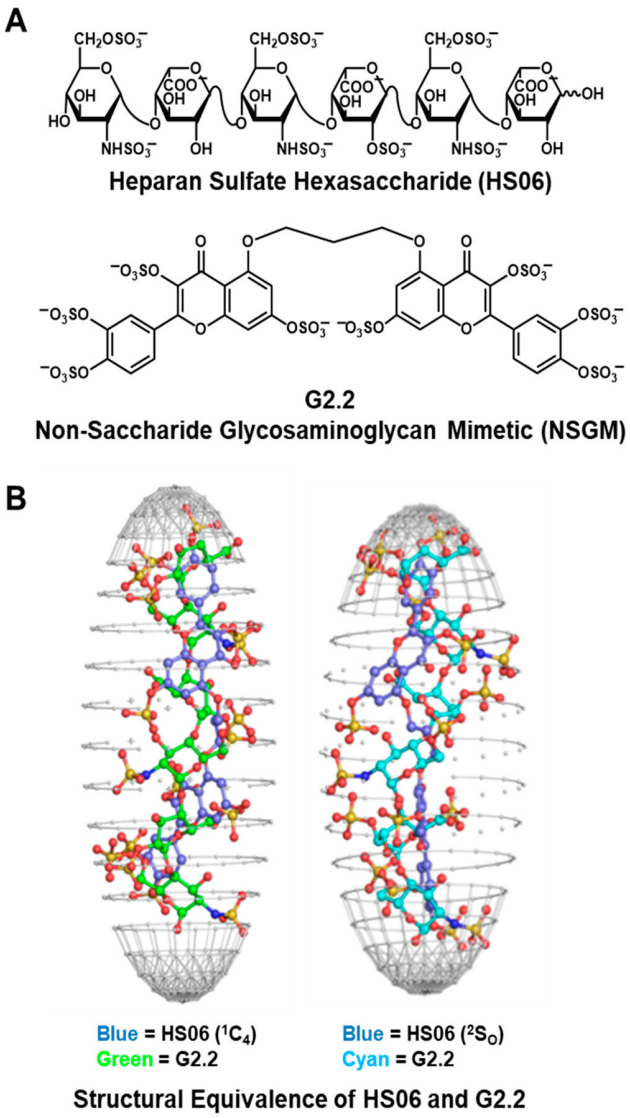


Figure S1. GAG recognition of proteins mimicked by non-saccharide GAG mimetics (NSGMs). (A) Structures of HS06, a GAG sequence, and G2.2, a representative NSGM. (B) Three-dimensional overlay of HS06 and G2.2 shows significant overlap of a number of sulfate groups, which determine protein recognition and biological activity. For further details on glycosaminoglycan mimicking potential of synthetic, homogenous, sulfated small molecules see B. Nagarajan, N. V. Sankaranarayanan, B. B. Patel and U. R. Desai, *PLoS One*, **2017**, 12, e0171619.

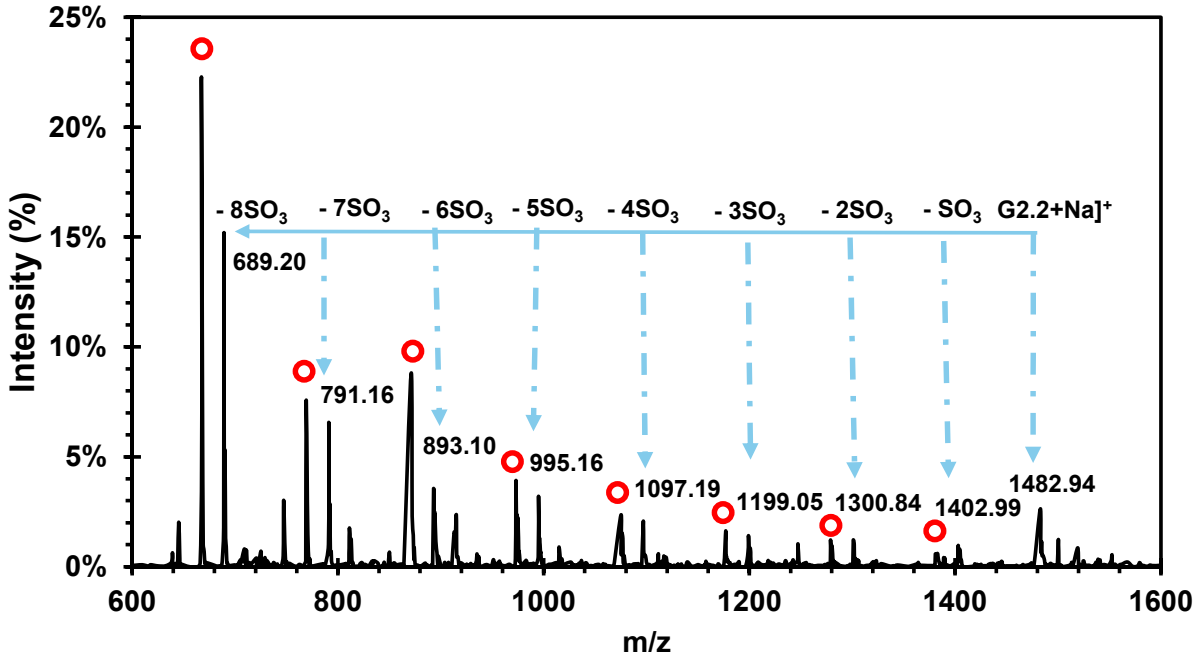
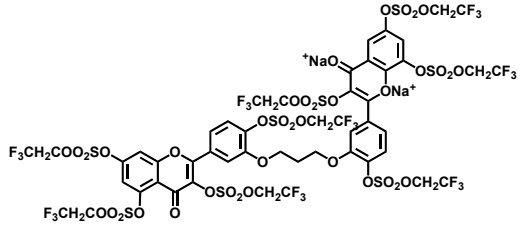
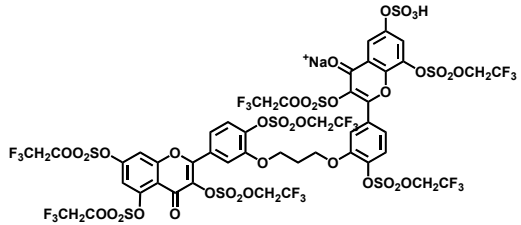
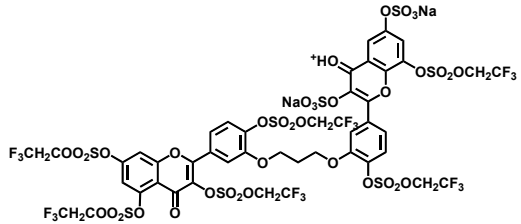
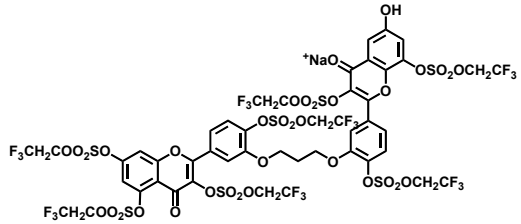
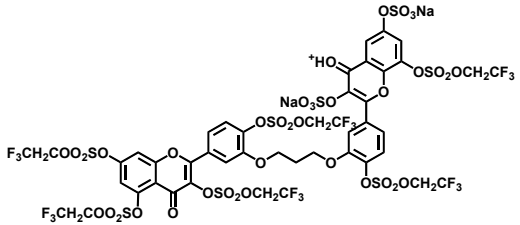
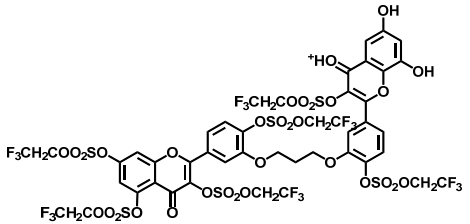
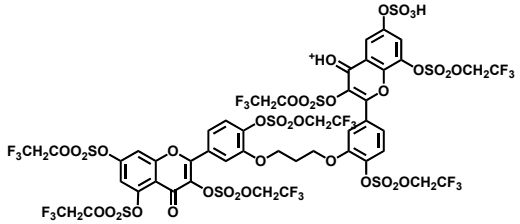
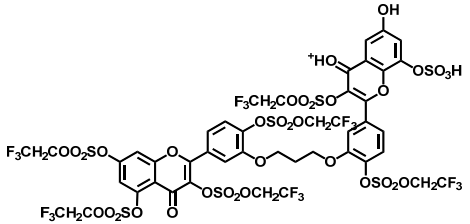
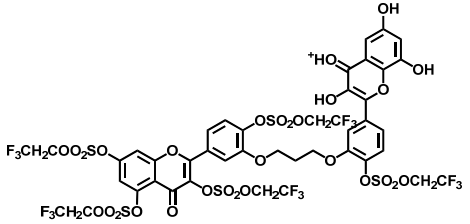
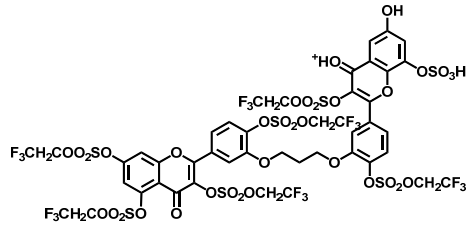


Figure S2. ESI-MS of the G2.2 peak eluting at 0.3 min in the void volume (Fig. 2A). Sodiated molecular ions are indicated by dashed arrows, while protonated ions are marked by a red circle. Signals corresponding to *in-situ* sulfate loss are detected under ESI conditions indicating sulfate lability, which gives rise to chromatographic complexity.

Table S1. ESI-MS (m/z) values for peaks **a**—**j** (Fig. 2A).

Peak	RT [a] (min)	Primary Peak (m/z)	Charge State	Theoretical Mass (m/z)	Corresponding Species Formula	Example Species Structure[b]
a	9.6	991.25	2+	992.89	**$[eG2.2 + 2Na]^{2+}$**	
b	9.4	1879.30	1+	1880.78	**$[eG2.2 – 1CH_2CF_3 + 1H + Na]^{1+}$**	
c	9.3	1820.24	1+	1820.76	**$[eG2.2 – 2CH_2CF_3 + 2Na + Na]^{1+}$**	
d	9.2	1801.28	1+	1800.83	**$[eG2.2 – 1SO_3CH_2CF_3 + 1H + Na]^{1+}$**	

e	9.1	1820.44	1+	1820.76	**[eG2.2 – 2CH_2CF_3 + 2Na + Na]$^{1+}$**	
f	8.9	1617.32	1+	1616.88	**[eG2.2 – 2$SO_3CH_2CF_3$ + 2H + H]$^{1+}$**	
g	8.8	1859.31	1+	1858.79	**[eG2.2 – 1CH_2CF_3 + 1H + H]$^{1+}$**	
h	8.6	1697.43	1+	1696.84	**[eG2.2 – 1$SO_3CH_2CF_3$ – 1CH_2CF_3 + 2H + H]$^{1+}$**	
i	8.4	1455.29	1+	1454.92	**[eG2.2 – 3$SO_3CH_2CF_3$ + 3H + H]$^{1+}$**	

j	8.3	1697.23	1+	1696.84	**[eG2.2 – 1$SO_3CH_2CF_3$ – 1CH_2CF_3 + 2H + H]$^{1+}$**	

[a] *R_T refers to retention time of the corresponding peak in liquid chromatogram.*

[b] *Structures shown refer to example species (not the only species that correspond to the mass peak).*

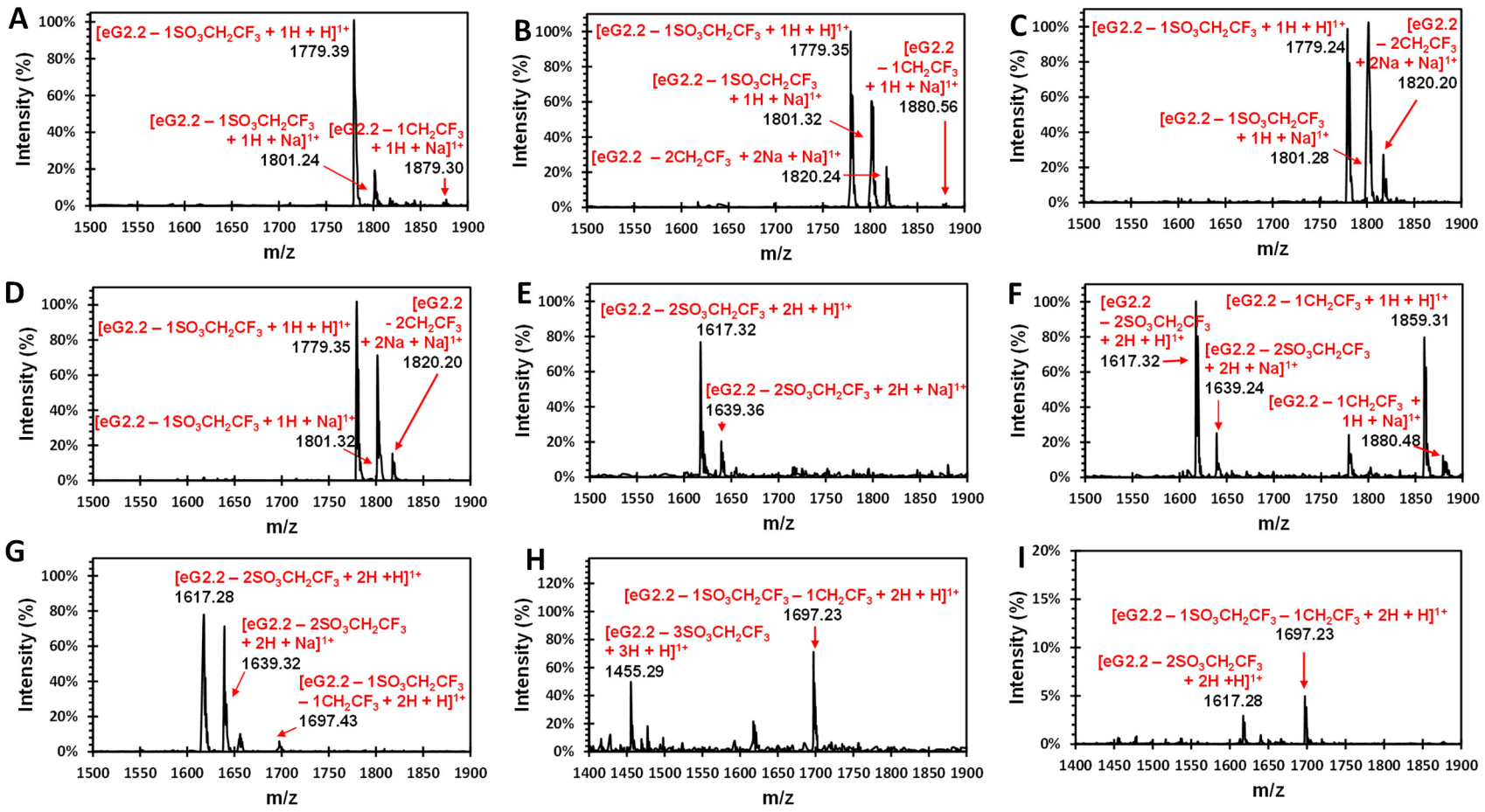


Fig. S3. ESI-MS spectra corresponding to chromatographic peaks **b** to **j** eluting between 8.3 and 9.6 minutes, as shown in figure 1B. Panels A to I represent the ESI-MS spectra of peaks **b** to **j**, respectively, in the order of their elution. These spectra illustrate the mass-to-charge (m/z) profiles of the variably esterified and desulfated species identified from LC-MS analysis.

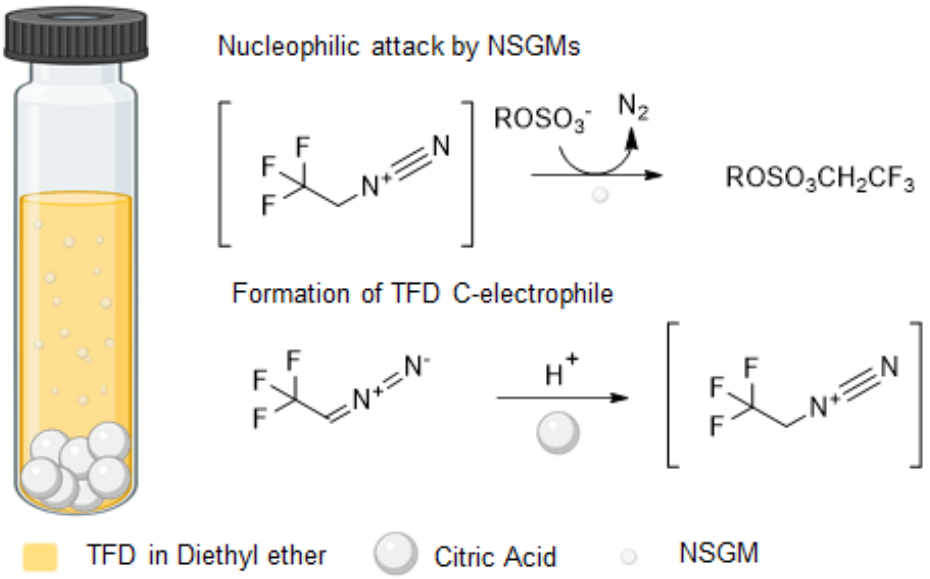


Figure S4. Solid-liquid interface hypothesis for chemical derivatization using TFD.

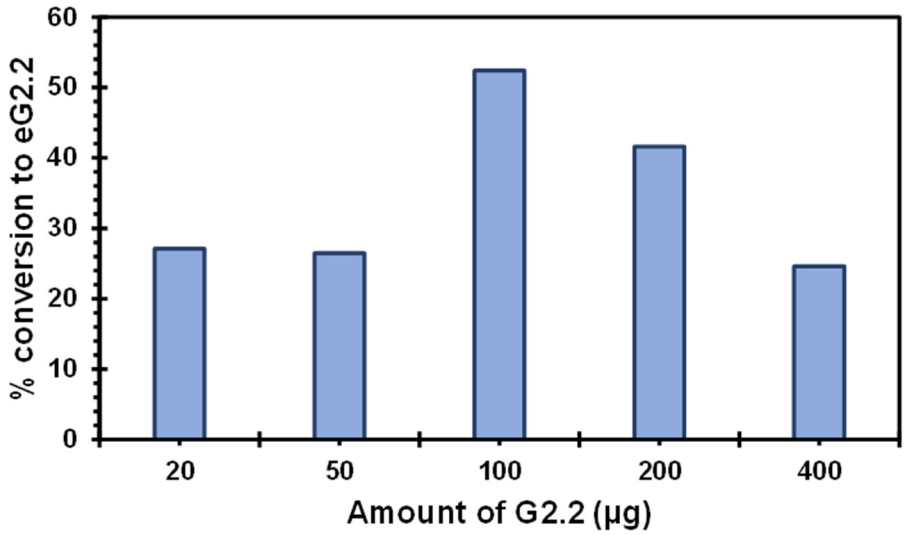
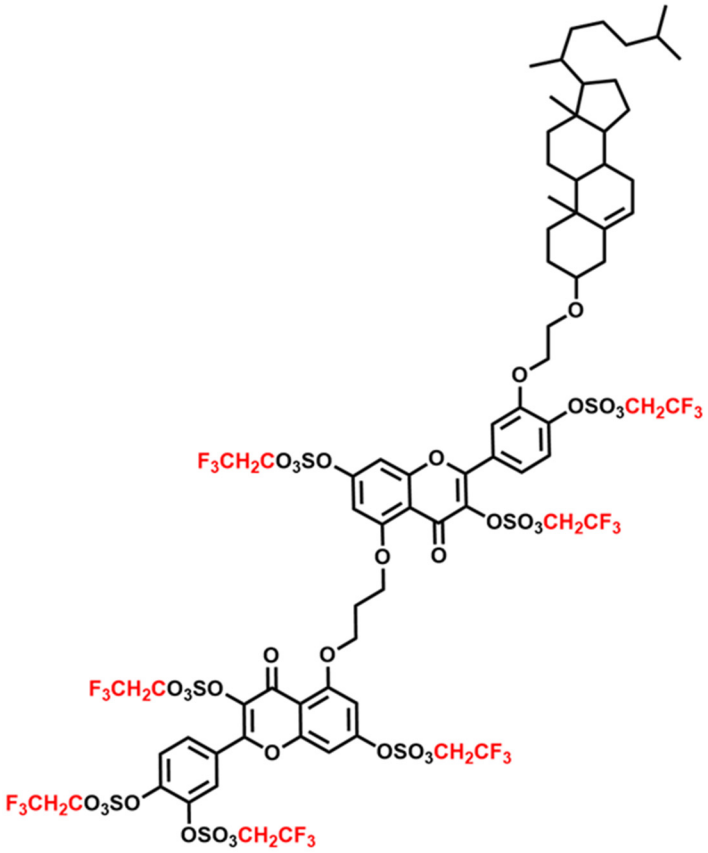


Figure S5. Maximum conversion of **G2.2** to **eG2.2**. To test the solid-liquid interface hypothesis and determine the minimum substrate threshold required for maximum effective esterification, varying amounts of **G2.2** (20 to 400 µg) were prepared by lyophilizing corresponding volumes of 1 mM aqueous **G2.2** solution. Each dried sample was subjected to esterification under the standard conditions with proportional amounts of anhydrous citric acid. After 24 hours, aliquots were collected, dried using speed vac, reconstituted in methanol: water (4:1) and analyzed by UPLC-MS.



eG2C

Chemical Formula: $C_{76}H_{79}F_{21}O_{36}S_7$
Exact Mass: 2190.2061

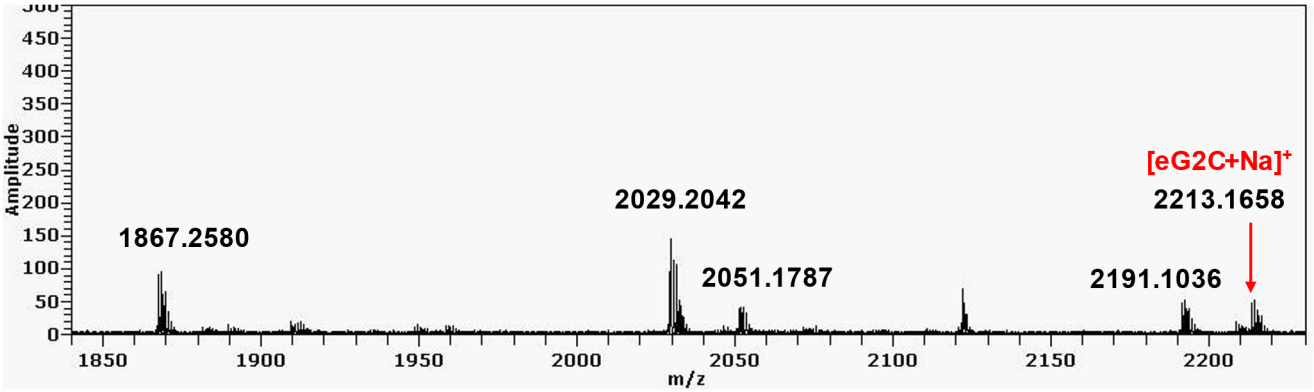
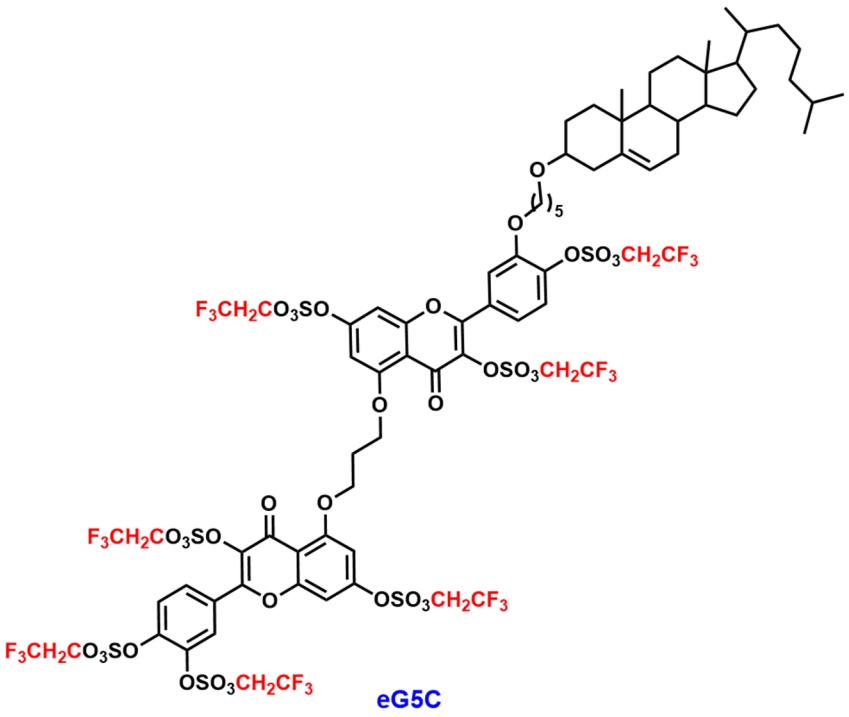


Figure S6. HRMS spectrum of eG2C.



Chemical Formula: $C_{79}H_{85}F_{21}O_{36}S_7$
Exact Mass: 2232.2530

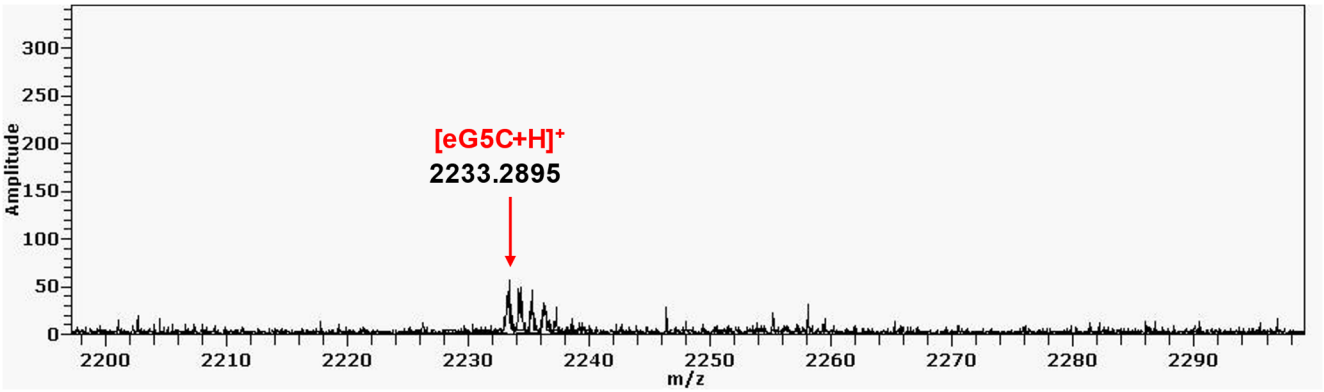
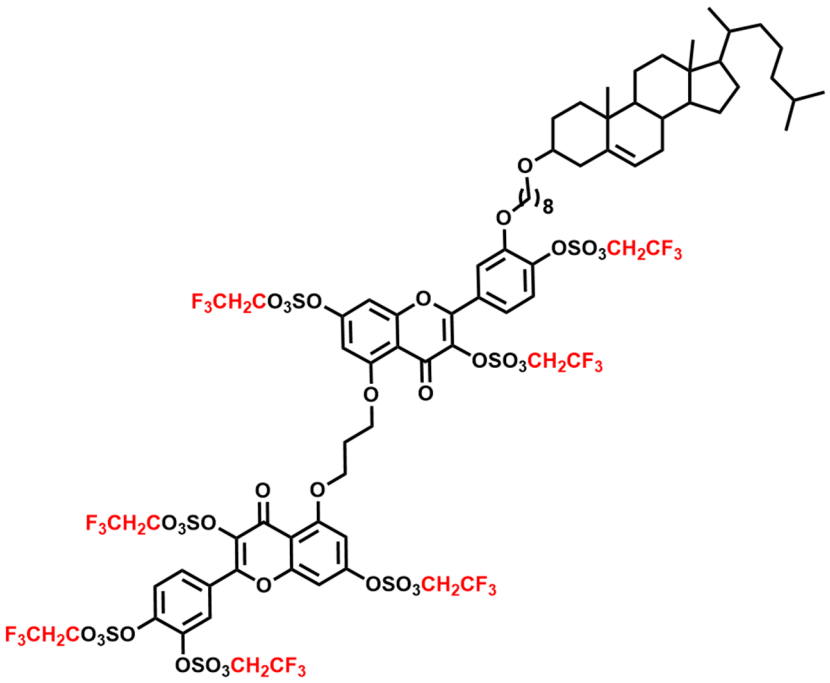


Figure S7. HRMS spectrum of eG5C.



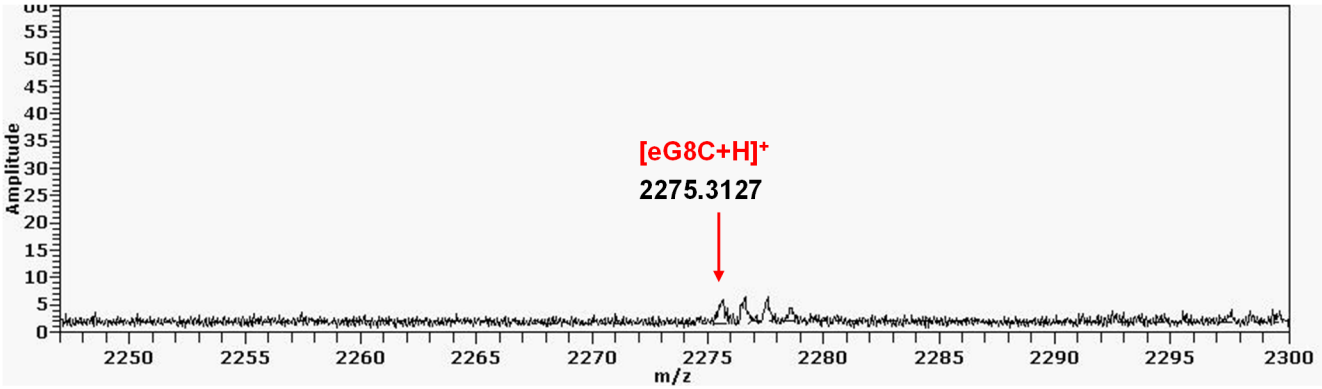
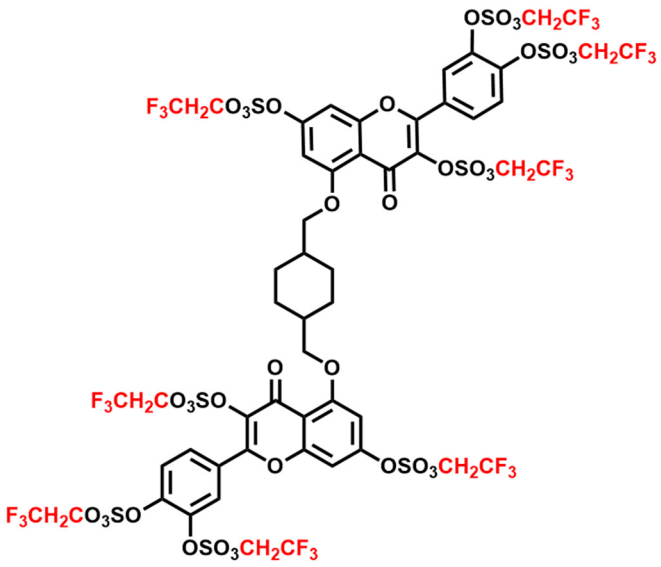


Figure S8. HRMS spectrum of eG8C.



eMQD1

Chemical Formula: $C_{54}H_{40}F_{24}O_{38}S_8$
Exact Mass: 2007.8580

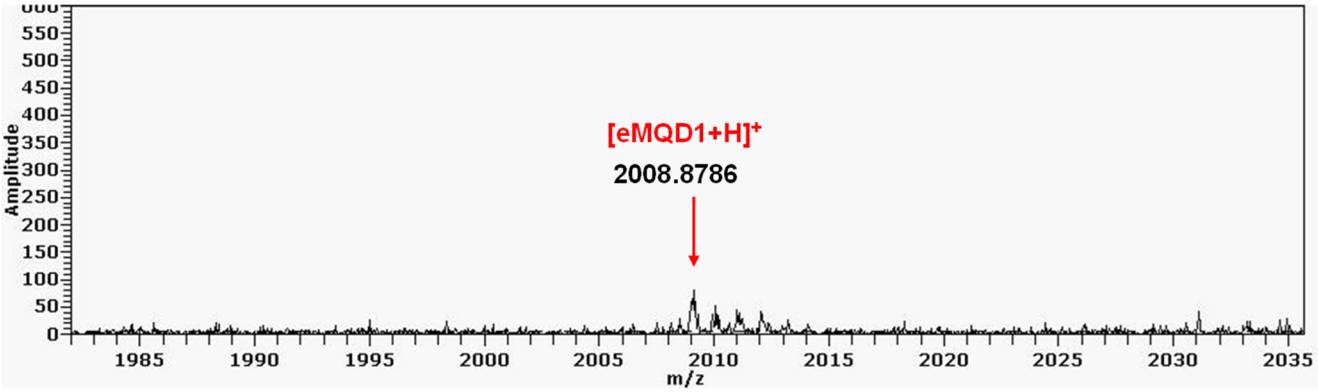


Figure S9. HRMS spectrum of eMQD1.

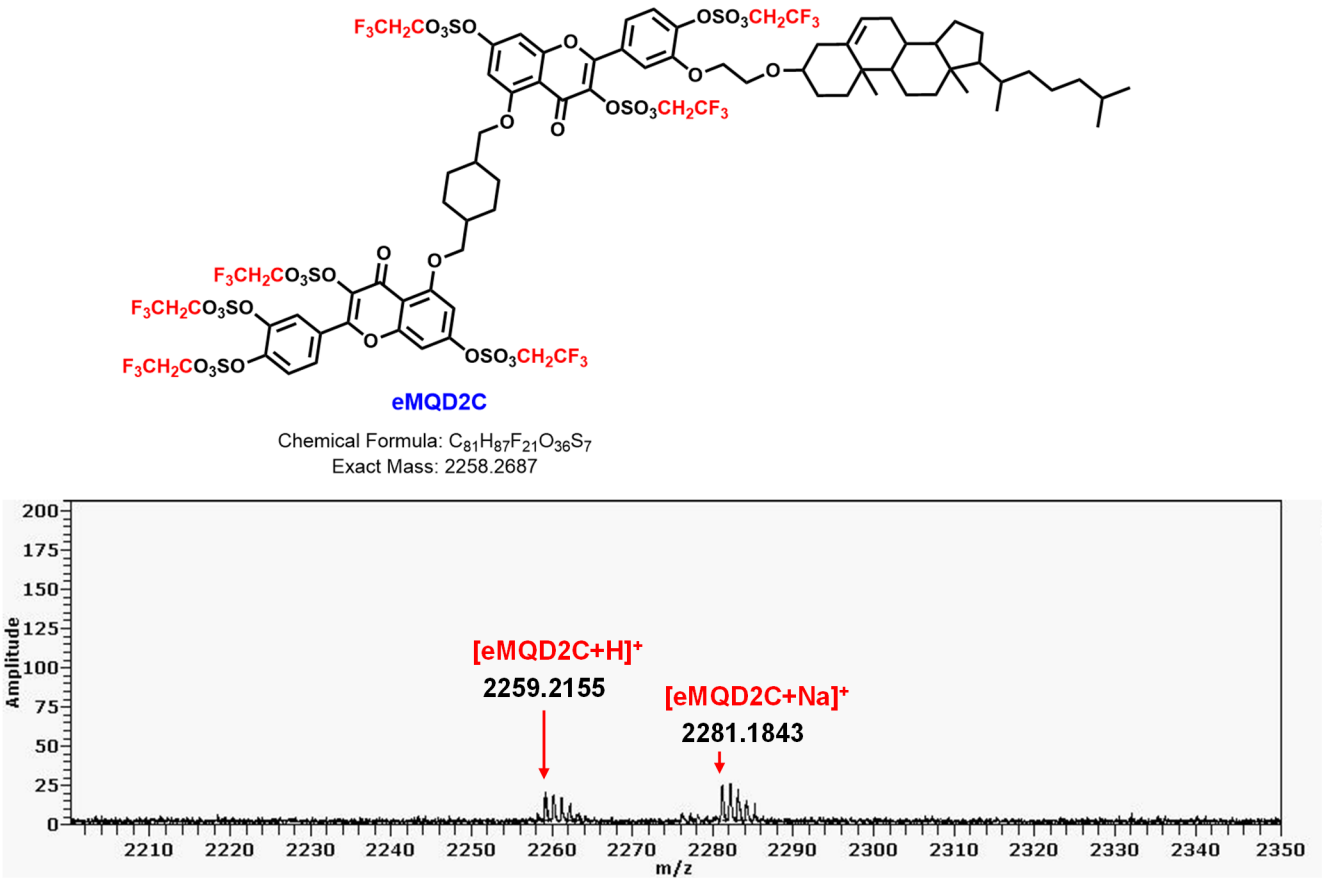


Figure S10. HRMS spectrum of eMQD2C.

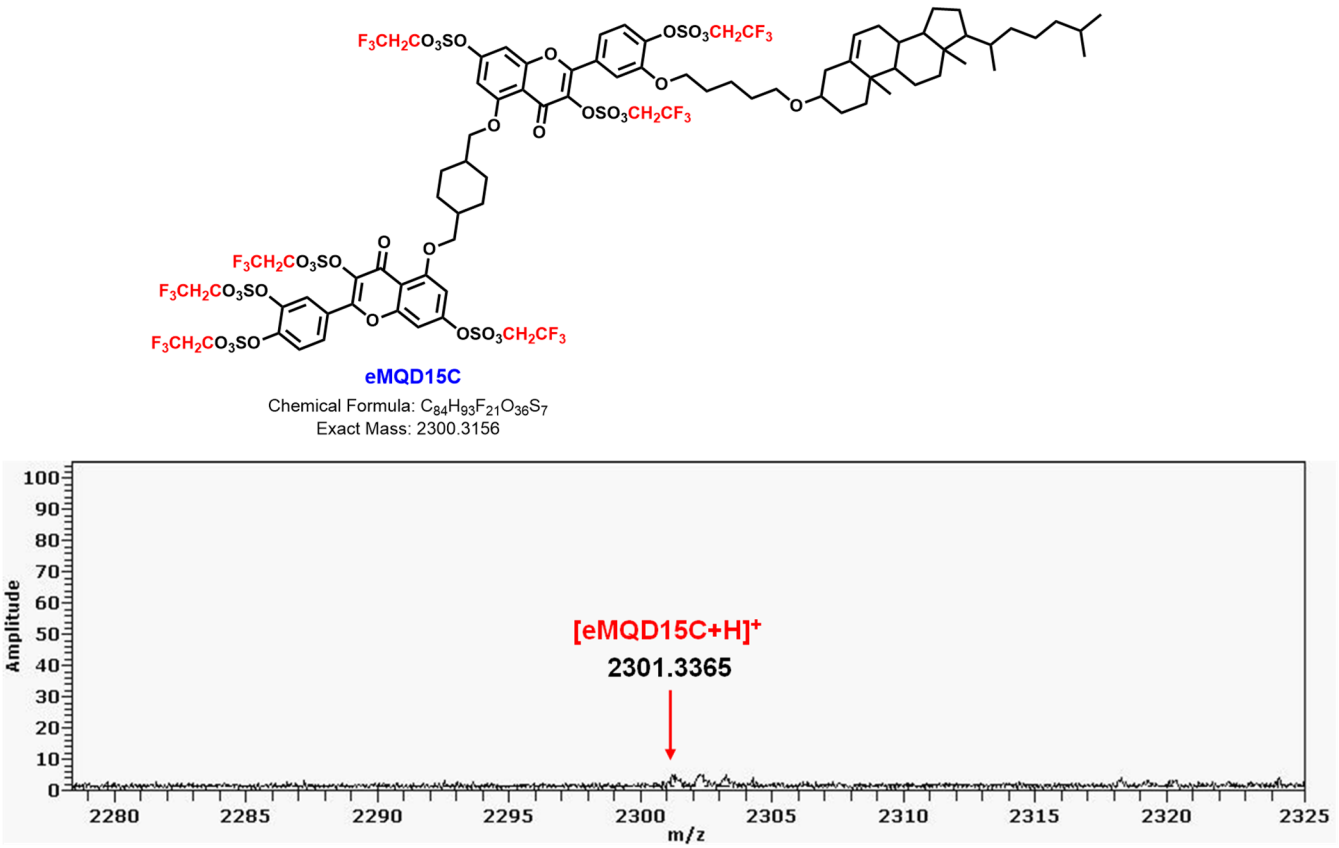
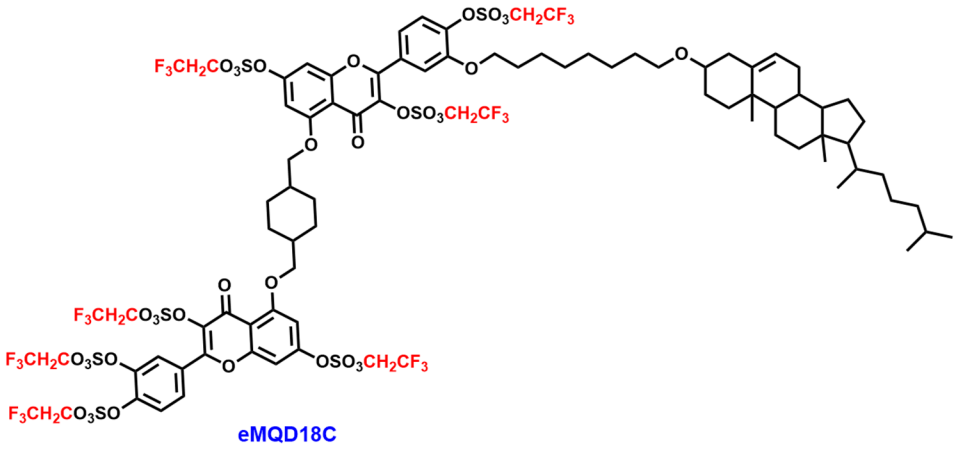


Figure S11. HRMS spectrum of eMQD15C.



eMQD18C

Chemical Formula: $C_{87}H_{99}F_{21}O_{36}S_7$
Exact Mass: 2342.3626

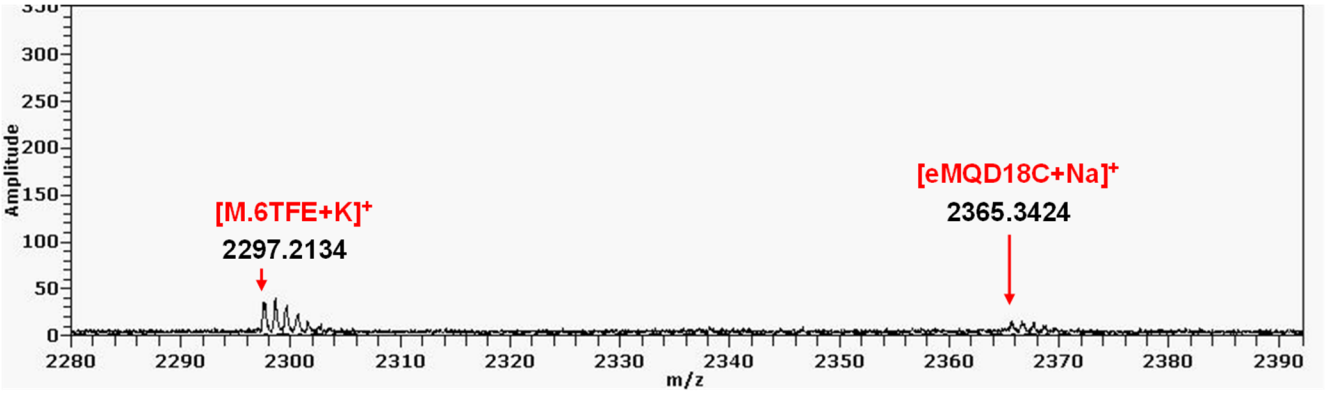


Figure S12. HRMS spectrum of eMQD18C.