Synthesis of a Fullerene Derivative for the Inhibition of HIV Enzymes

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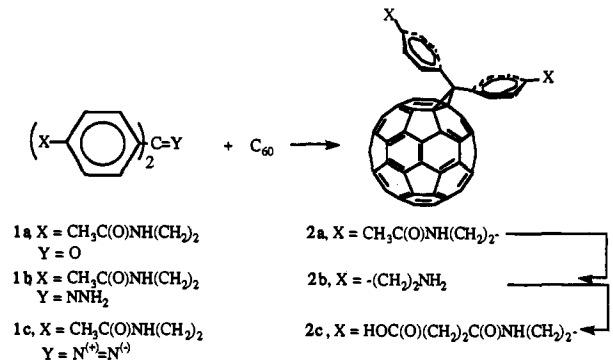
Abstract: A diamido diacid diphenyl fulleroid derivative (**2c**) was designed specifically to inhibit an HIV enzyme. The detailed synthesis and mass spectrometric analysis of the water-soluble, biologically active methanofullerene are described. The compound was prepared in three steps from C_{60} via a suitably substituted diphenyldiazomethane. High-resolution mass spectrometric analysis was possible only under mild matrix-assisted laser desorption/ionization Fourier transform mass spectrometry conditions. Direct infrared or ultraviolet laser desorption resulted exclusively in observation of C_{60} ions, in either positive or negative mode.

In this paper, we give the detailed synthesis and mass spectrometric analysis of a water-soluble, biologically active diamido diacid diphenyl fulleroid derivative (**2b**). The target compound was specifically designed to inhibit certain HIV enzymes: protease (HIVP)[1] and reverse transcriptase (HIVRT).[2] Design considerations required that this compound have polar functional groups at one end of the carbon cluster and, ideally, cationic moieties for the protease and anionic sites for the reverse transcriptase.[1]

There exist a variety of procedures for C_{60} functionalization,[3] but, to date, a specifically targeted molecule, with a special function in mind, was not prepared. Among the various methods available, functionalization via cycloaddition is by far the simplest and most versatile.[4,5] It was decided that we would build a fulleroid derived from diphenyldiazomethane since we were familiar with its reactivity toward C_{60} and the synthesis of the precursor benzophenone derivatives is generally facile. The target methanofullerene derivative was prepared according to Scheme I.

Scheme I



The substituted diphenyldiazomethane was prepared in the usual manner from the substituted benzophenone hydrazone by oxidation with nickel peroxide. The formation of **2a** from buckminsterfullerene C_{60}[6] followed the usual methanofullerene synthesis method.[4] The bis(acetamide) was hydrolyzed in acetic acid/aqueous hydrochloric acid and converted to bis(succinamide) **2c** by treatment with succinic anhydride. Compounds **2a–c** exhibit the usual methanofullerene properties. Compound **2c** is soluble in water at pH ≥ 7, making it an ideal substrate for evaluation of physiological and pharmaceutical properties of a methanofullerene.

The only difficult step in the synthesis was the hydrolysis of bis(acetamide) **2a** which we found to be extremely sluggish, requiring workup after ca. 16 h followed by repeated submittal of the unreacted **2a** to hydrolytic conditions. Many different combinations of solvents and acids were tried, and the conditions given in the Experimental Section are, so far, the most reproducible.

Upon obtaining **2b**, we assumed its conjugate acid would be a good target for inhibition of HIVP but were disappointed when we discovered that its hydrochloride salt was not soluble in water.[7]

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(1) Friedman, S. H.; Kenyon, G.; DeCamp, D. L.; Sijbesma, R. P.; Srdanov, G.; Wudl, F. *J. Am. Chem. Soc.*, preceding paper in this issue.

(2) Schinazi, R. F.; Sijbesma, R.; Srdanov, G.; Hill, C. L.; Wudl, F. Submitted for publication.

(3) *Fullerenes Synthesis, Properties, and Chemistry of Large Carbon Clusters*; Hammond, G., Kuck, V. J., Eds.; ACS Symposium Series 481; American Chemical Society: Washington, DC, 1992. See entire issue no. 3 of *Acc. Chem. Res.* **1992**, *25*. Hirsch, A.; Soi, A.; Karfunkel, H. R. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 766. Chiang, L. Y.; Miller, G. *J. Am. Chem. Soc.* **1992**, *114*, 10154. Chiang, L. Y.; Swirczewski, J. W.; Hsu, C. S.; Chowdhury, S. K.; Cameron, S.; Creegan, K. *J. Chem. Soc., Chem. Commun.* **1992**, 1790.

(4) (a) Suzuki, T.; Li, Q.; Khemani, K. C.; Wudl, F.; Almarsson, Ö. *Science* **1991**, *254*, 1186. (b) Wudl, F. *Acc. Chem. Res.* **1992**, *25*, 157. (c) Suzuki, T.; Li, Q.; Khemani, K.; Wudl, F.; Almarsson, Ö. *J. Am. Chem. Soc.* **1992**, *114*, 7301. The product of diaryldiazomethane addition is usually a mixture of isomers, as detected by NMR. The mixture is usually heated overnight in *o*-dichlorobenzene to convert it to a single, thermodynamically most stable isomer (see Experimental Section).

(5) Rubin, Y. *J. Am. Chem. Soc.* **1993**, *115*, 344. Prato, M.; Suzuki, T.; Foroudian, H.; Li, Q.; Khemani, K. C.; Wudl, F.; Leonetti, J.; Little, R. D.; White, T.; Rickborn, B.; Yamago, S.; Nakamura, E. *J. Am. Chem. Soc.* **1993**, *115*, in press.

(6) Krätschmer, W.; Lamb, L. D.; Fostiropoulos, K.; Huffman, D. R. *Nature* **1990**, *347*, 354. Haufler, R. E.; Conceicao, J.; Chibante, L. P. F.; Chai, Y.; Byrne, N. E.; Flanagan, S.; Haley, M. M.; O'Brien, S. C.; Pan, C.; Xiao, Z.; Billups, W. E.; Ciufolini, M. A.; Hauge, R. H.; Margrave, J. L.; Wilson, L. J.; Curl, R. F.; Smalley, R. E. *J. Phys. Chem.* **1990**, *94*, 8634. For a review, see entire issue no. 3 of *Acc. Chem. Res.* **1992**, *25*.

(7) Later, we found that the tosylate salt is actually water-soluble. This is a counterintuitive result since salts where both ions are large are usually water-insoluble and salts of large cations with small anions (and vice versa) are more water-soluble, hence, the surprise at water insolubility of the chloride.

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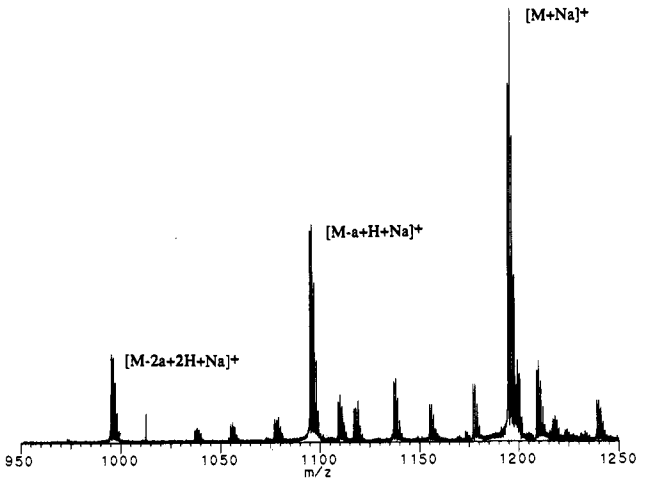

Figure 1. High-resolution positive ion MALDI Fourier transform mass spectrum of **2c**, where a = $COCH_2CH_2COOH$.

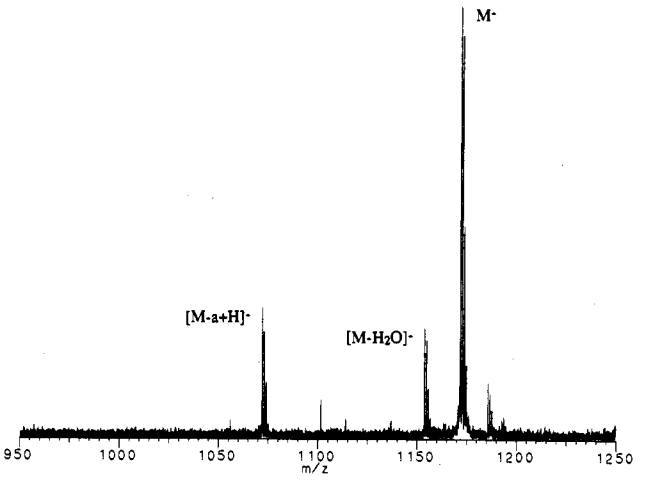

Figure 2. High-resolution negative ion MALDI Fourier transform mass spectrum of **2c**, where a = $COCH_2CH_2COOH$.

These and other negative results[8] led us to try the reaction of **2b** with succinic anhydride, yielding **2c**, whose bis sodium salt was sparingly soluble in water (ca. 1 mg/mL) and expected to inhibit (HIVRT).[2]

Structural Characterization. The infrared, UV–vis, and ^1H NMR spectra were in good agreement with the proposed structure (see Experimental Section). At the time the research on this molecule was in progress, it was discovered that a number of fulleroids actually have a cyclopropane (ring-closed, "methanofullerene") rather than an annulene (ring-opened, "fulleroid") structure.[9] We assigned **2b** the methanofullerene structure shown in the scheme, based on (1) the ^{13}C NMR resonance of the bridgehead atoms of **2b** appearing at 79.37 ppm (the resonances of the bridgehead carbons for methanofullerenes are in the 77–80 ppm region, while those of the fulleroids are in the 137–150 ppm region)[9] and (2) the UV–vis spectra of **2a–c** all exhibiting the diagnostic 430-nm peak of methanofullerenes.[9]

Because the quantities available for analysis were too small for traditional elemental analysis, mass spectrometry was used for assessment of elemental composition. Several attempts at fast atom bombardment mass spectrometry (FABMS) failed to produce spectra with a molecular ion peak; the only observable peaks were due to C_{60}. Similarly, direct laser desorption Fourier transform mass spectrometry (FTMS), using either pulsed carbon dioxide laser desorption[10] or ultraviolet laser desorption, yielded spectra containing only peaks due to C_{60}^+ ions. However, the somewhat gentler technique of matrix-assisted laser desorption did provide the requisite analytical information described below.

High-Resolution Mass Spectra. Figure 1 contains the high-resolution MALDI-FTMS (matrix-assisted laser desorption/ionization Fourier transform mass spectra)[11] positive ion spectrum of compound **2c**. As expected, almost all of the ions observed are sodium attachment ions. The most abundant ion is the molecular sodium attachment ion $[M^+Na]^+$, with m/z 1195.2. The second and third most abundant ions correspond to loss of either one or two $COCH_2CH_2COOH$ groups. Similarly, the most abundant ion in the negative ion spectrum (Figure 2) is the M^- ion, with m/z 1171.8. The other two most abundant ions correspond to loss of H_2O and loss of a $COCH_2CH_2COOH$ fragment. Mass resolution of approximately 8000 is obtained for the positive ion spectrum and resolution of about 9000 for the negative ion spectrum.

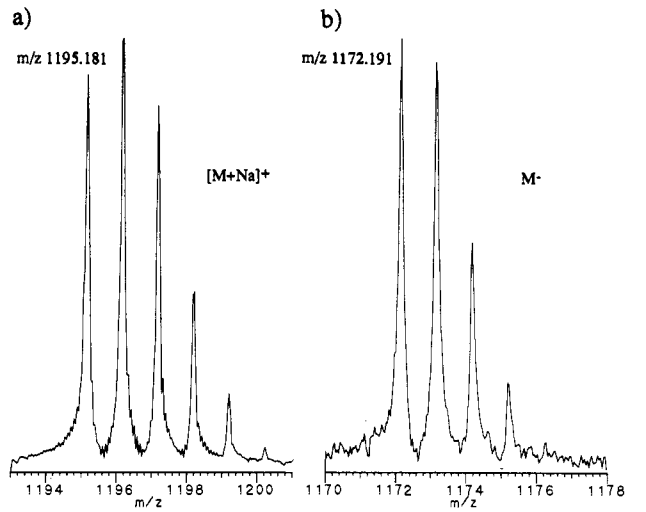

Figure 3. Accurate mass molecular ion measurements of **2c**: (a) $[M^+Na]^+$, polyethyleneglycol-1000 as internal calibrant and (b) M^-, 2,4,6-tris(perfluoroheptyl)-1,3,5-triazine as internal calibrant.

Molecular Ion Mass Determination. In another set of measurements, with the appropriate internal calibrants added, an average mass measurement difference of 2.1 ppm from the calculated mass of the all ^{12}C molecular ion species $[M^+Na]^+$ was measured for four separate spectra, each obtained by averaging the spectra resulting from nine laser shots. For the negative molecular ion, M^-, an average mass accuracy of 7.1 ppm is obtained from all the ^{12}C ions determined from three spectra, each resulting from time domain addition of 27 spectra acquired using the corresponding number of laser shots. Figures 3a and 3b show typical mass measurement accuracy results for the molecular ion region of the positive and negative ion spectra. No peaks attributable to C_{60} ions are seen in either positive ion or negative ion MALDI spectra.

Conclusions

Samples for pharmacological evaluation were submitted to research groups at University of California, San Francisco,[1] and Emory University. The results were quite encouraging. Compound **2c** was found, as predicted,[1] to be an inhibitor of HIVP. It was also an inhibitor of HIV-1 and HIV-2 reverse transcriptase in the low micromolar concentration range.[2] The compound was also found to be nontoxic to three different test cell lines.[2] This

(8) The *N,N,N',N'*-tetramethyl derivative of **2b** hydrochloride was *also insoluble in water*. Finally, reaction of the *N,N,N',N'*-tetramethyl derivative with propane sultone afforded a zwitterion which was also *insoluble in water*.

(9) Prato, M.; Maggini, M.; Stimpf, E.; Scorrano, G.; Lucchini, V.; Eiermann, M.; Wudl, F. *J. Am. Chem. Soc.*, in press.

(10) Wilkins, C. L.; Weil, D. A.; Yang, C. L. C.; Ijames, C. F. *Anal. Chem.* **1985**, *57*, 520–524.

(11) Castoro, J. A.; Köster, C.; Wilkins, C.; *Rapid Commun. Mass Spectrom.* **1992**, *6*, 239–241.

is clearly the "first generation" of methanofullerenes to be tested, and further structural modification should produce considerably more potent retroviral enzyme inhibitors.

Since compound **2b** is a key intermediate, we are currently pursuing further derivatization to produce water-soluble cationic derivatives to specifically inhibit HIVP.

From the mass spectral experiments, it is clear that methanofullerene derivatives are readily converted to C_{60} under FABMS or direct laser desorption conditions but that use of MALDI-FTMS can suppress this undesired decomposition and provide analytical data on the unchanged analyte. Thus, it is extremely important to use the appropriate mass spectral technique if reliable conclusions are to be drawn regarding structures of putative fulleroids and methanofullerenes.

Experimental Section

***N*-Acetyl-2-phenylethylamine.** This compound was prepared from 2-phenylethylamine and acetic anhydride according to a literature procedure.[12]

4,4'-Bis(*N*-acetyl-2-aminoethyl)benzophenone (1a). Compound **1** (3.95 g, 24.2 mmol) was dissolved in CCl_4 (80 mL). The solution was cooled in an ice bath, 13.4 g (100 mmol) of $AlCl_3$ was added slowly, and the resulting slurry was stirred for 12 h at room temperature. The reaction mixture was poured into ice-cold 2 N aqueous HCl, made basic with concentrated KOH solution, and extracted with CH_2Cl_2 (400 mL). The organic layer was evaporated, and the crude product was purified by column chromatography (silica, CH_2Cl_2/MeOH, 95:5 v/v). Yield: 3.7 g (82%) of **2**. A sample was recrystallized from toluene/methanol: mp 176–178 °C; HRMS *m/z* calcd for $C_{26}H_{24}N_2O_2$ 352.179, found 352.1823. ^{1}H NMR ($CDCl_3$): δ 7.73, 7.31 (2 d, 8H, J = 8 Hz, arom H), 5.79 (br s, 2H, NH), 3.54 (m, 4H, CH_2CH_2N), 2.90 (t, 4H, $PhCH_2CH_2N$), 1.97 (s, 6H, $COCH_3$). IR (KBr): 3280 s, 3060 m, 2910–2850 m, 1635 s, 1545 s, 1280 s cm^{-1}.

4,4'-Bis(*N*-acetyl-2-aminoethyl)benzophenone Hydrazone (1b). Compound **2** (430 mg, 1.22 mmol) was dissolved in dry ethanol (70 mL). Dry hydrazine (3.5 mL) and acetic acid (7 mL) were added, and the reaction mixture was allowed to reflux for 1.5 h. The solvents were evaporated *in vacuo*, and the product was purified by column chromatography (neutral alumina, CH_2Cl_2/MeOH, 98:2 v/v). Yield: 290 mg (66%) of **3** as a glassy solid. ^{1}H NMR ($CDCl_3$): 7.1–7.43 (m, 8H, arom H), 5.72 and 5.57 (2 br s, 2H, NH), 3.4–3.65 (m, 4H, CH_2CH_2N), 2.70–2.95 (4H, $PhCH_2CH_2N$), 1.93 and 1.99 (2 s, 6H, $COCH_3$). HRMS (EI): *m/z* calcd 366.2056, found 366.2068.

4,4'-Bis(*N*-acetyl-2-aminoethyl)diphenyldiazomethane (1c). Hydrazone **1b** (32 mg, 0.086 mmol) was dissolved in 20 mL of freshly distilled THF. One drop of a saturated solution of NaOH in EtOH and 51 mg of nickel peroxide were added. The mixture was stirred over molecular sieves (4 Å) until all the hydrazone had disappeared and one red spot was visible on TLC (1.5 h). The solution was filtered over a Celite pad and used directly for the next step. ^{1}H NMR ($CDCl_3$): 7.23 (s, 8H, arom H), 5.71 (br s, 2H, NH), 3.52 (m, 4H, CH_2CH_2N), 2.83 (4H, $PhCH_2CH_2N$), 1.96 (s, 6H, $COCH_3$). IR (neat): 3280, 3090, 2040, 1645, 1545, 1440, 1290 cm^{-1}. UV–vis (THF): 533, 288, 266 nm.

4,4'-Bis(*N*-acetyl-2-aminoethyl)diphenyl C_{61} (2a). To a solution of C_{60} (100 mg, 0.139 mmol) in toluene (400 mL) was added a solution of **1c** (50 mg, 0.137 mmol) in THF (70 mL). The mixture was stirred overnight. The solvent was removed, and the product was purified by column chromatography (silica, toluene/MeOH, 93:7 v/v). The purified product was heated for 16 h in refluxing *o*-dichlorobenzene. The solvent was removed *in vacuo*, and traces of solvent were removed by precipitation with methanol from a toluene/methanol solution. Yield: 55 mg (38%) of **2a** (75% based on consumed C_{60}). ^{1}H NMR ($CDCl_3$/CD_3OD): 8.06, 7.34 (2 d, 8H, arom H), 6.72 (br s, 2H, NH), 3.48 (t, 4H, CH_2CH_2N), 2.87 (4H, $PhCH_2CH_2N$), 1.95 (s, 6H, $COCH_3$). IR (KBr): 3280 br, 2930 m, 1655 s, 1550 s, 1432 s, 1369 m, 1291 m, 1192 m, 598 w, 581 w, 568 w, 532 s cm^{-1}. FABMS (*m*-nitrobenzyl alcohol): *m/z* 1057 (M + H)$^+$, 720 (C_{60}^+). Anal. Calcd for $C_{81}H_{24}N_2O_2H_2O$: C, 90.49; H, 2.34; N, 2.60. Found: C, 90.93; H, 2.55; N, 2.39.

4,4'-Bis(2-aminoethyl)diphenyl C_{61} (2b). A solution of 25.7 mg of **2a** in acetic acid (7.5 mL) and concentrated aqueous HCl (2 mL) was allowed to reflux overnight. The solvent was evaporated *in vacuo* to afford the product as its bis(hydrochloride). Yield: 25.0 mg (98%). ^{1}H NMR (CD_3OD/CS_2): 8.21, 7.43 (2 d, 8H, arom H), 3.18 (t, 4H, CH_2CH_2N), 3.01 (4H, $PhCH_2CH_2N$). IR (KBr): 3400 br, 3020 m, 2915 m, 1608 s, 1505 s, 1468 s, 1430 s, 1385 m, 1320 w, 1245 w, 1190 m, 1180 sh, 1125, 1090, 1020, 960, 900, 815 sh, 800, 748, 715, 615, 590 w, 580 w, 560 w, 530 s cm^{-1}. FABMS (*m*-nitrobenzyl alcohol): *m/z* 973 (M + H)$^+$, 720 (C_{60}^+).

Water-Soluble C_{60} Derivative (2c). To 25 mg of **2b**·2 HCl (0.024 mmol) was added 103 mg (1.02 mmol) of succinic anhydride in 10 mL of dry pyridine. The resulting red solution was stirred overnight. The reaction mixture was poured into 2 N aqueous HCl (100 mL) and centrifuged. The precipitate was washed twice with water and dissolved in 25 mL of 0.1 N aqueous NaOH. The solution was centrifuged to remove insoluble side products, and the supernatant was acidified with concentrated aqueous HCl. The resulting precipitate was centrifuged, washed with water and methanol, and finally dried *in vacuo*. Yield: 25.7 mg (93%). ^{1}H NMR (CD_3OD/CS_2): 8.10, 7.36 (2 d, 8H, arom H), 3.44 (t, 4H, CH_2CH_2N), 2.86 (t, 4H, $PhCH_2CH_2N$), 2.55 and 2.43 (2 t, 8H, $COCH_2CH_2CO$). ^{13}C NMR (DMSO-d_6, 125 MHz): d 173.78, 170.78, 148.93, 145.73, 144.58, 144.52, 144.24, 144.09, 143.98, 143.60, 143.30, 142.36, 142.30, 141.66, 141.59, 139.97, 139.41, 137.13, 136.75, 130.94, 128.90, 79.37, 51.24, 34.87, 30.01, 29.14. IR (KBr): 3425 br, 2925 m, 1706 s, 1650 s, 1550, 1427, 1190, 590 m, 575 m, 557 m, 526 s cm^{-1}. UV–vis (CS_2/MeOH) λ_{max} (nm): 363, 433 (diagnostic for cyclopropane),[9] 500 (diagnostic for cyclopropane).[9] Reprecipitation with acid from base produced the MS sample which was pure by HPLC (column, VIDAC, C_{18} reverse phase; eluent, 10% MeOH/water; detector, variable wavelength, λ_{max} 370 nm; flow rate, 0.5 mL/min; retention time, 2.9–3.1 min (depending on pyridine concentration); impurity retention time, 5.87 min). Minor impurities with a dissimilar retention time to that of **2c** appear to be in the methanolic pyridine used for elution, as determined from a blank run (×100 gain). The pyridine salt in methanol shows no impurities within experimental error (machine integration, 100%).

Mass Spectrometry. Sample Preparation. Samples were prepared by mixing approximately 100 μg of analyte in a methanol:CS_2 solution (2:1 by volume) with 300 μL of a 50 mmol/L 2,5-dihydroxybenzoic acid (Fluka Chemical Co., Buchs, Switzerland) matrix solution containing 0.1% trifluoroacetic acid (Mallinckrodt, St. Louis, MO) in methanol and 30 μL of a 60 mmol/L aqueous NaCl solution. The resulting solutions were sprayed as aerosols onto a rotating stainless steel probe tip for homogenous deposition.

Matrix-assisted laser desorption/ionization (MALDI) Fourier transform mass spectra (FTMS)[11] were obtained with 357-nm radiation from a Lambda Physik (Göttingen, Germany) FL-2001 dye laser, pumped by a Lambda Physik EMG 201-MSC excimer laser (operating at 308 nm, 180 mJ/28 ns pulse) and a Millipore Extrel (Madison, WI) FTMS-2000 dual cell spectrometer equipped with a 7-T superconducting magnet. Spectra were obtained using a gated trapping sequence[11,13] with ejection of ions below *m/z* 750 and a 200-V peak-to-peak chirp excitation from 1 to 200 kHz at 180 Hz/μs sweep rate followed by detection. Each spectrum resulted from averaging between 2 and 27 time domain scans, acquiring 65 536 data points per scan. The averaged time domain data were augmented by an equal number of zeroes and base-line corrected prior to magnitude mode Fourier transformation. No apodization was used. Polyethyleneglycol-1000 was used as an external calibrant for the full spectra. Accurate mass measurements of molecular ion species were made by adding a small quantity of an internal calibrant to the sample solutions prior to deposition on the sample probe. Polyethyleneglycol-1000 served as an internal calibrant (9-point calibration) for $[M^+Na]^+$ mass determination, and 2,4,6-tris(perfluoroheptyl)-1,3,5-triazine (Fluka Chemical, Buchs, Switzerland) was used as an internal calibrant (2-point calibration, M^- and $[M - F]^-$) for analyte M^- mass determinations.

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(12) Smith, M. E.; Adkins, H. *J. Am. Chem. Soc.* **1938**, *50*, 657.

(13) Köster, C.; Castoro, J. A.; Wilkins, C. L. *J. Am. Chem. Soc.* **1992**, *114*, 7572–7574.