METHOD

SHAMS: Combining chemical modification of RNA with mass spectrometry to examine polypurine tract-containing RNA/DNA hybrids

KEVIN B. TURNER,¹ HYE YOUNG YI-BRUNOZZI,² ROBERT G. BRINSON,² JOHN P. MARINO,³ DANIELE FABRIS,¹ and STUART F.J. LE GRICE²

¹Department of Chemistry and Biochemistry, University of Maryland Baltimore County, Baltimore, Maryland 21250, USA

²RT Biochemistry Section, HIV Drug Resistance Program, National Cancer Institute, National Institutes of Health, Frederick, Maryland 21702, USA

³Center for Advanced Research in Biotechnology, University of Maryland Biotechnology Institute and National Institute of Standards and Technology, Rockville, Maryland 20850, USA

ABSTRACT

Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) has gained popularity as a facile method of examining RNA structure both *in vitro* and *in vivo*, exploiting accessibility of the ribose 2'-OH to acylation by *N*-methylisatoic anhydride (NMIA) in unpaired or flexible configurations. Subsequent primer extension terminates at the site of chemical modification, and these products are fractionated by high-resolution gel electrophoresis. When applying SHAPE to investigate structural features associated with the wild-type and analog-substituted polypurine tract (PPT)-containing RNA/DNA hybrids, their size (20–25 base pairs) rendered primer extension impractical. As an alternative method of detection, we reasoned that chemical modification could be combined with tandem mass spectrometry, relying on the mass increment of RNA fragments containing the NMIA adduct ($M_r = 133$ Da). Using this approach, we demonstrate both specific modification of the HIV-1 PPT RNA primer and variations in its acylation pattern induced by replacing template nucleotides with a non-hydrogen-bonding thymine isostere. Our selective 2'-hydroxyl acylation analyzed by mass spectrometry strategy (SHAMS) should find utility when examining the structure of small RNA fragments or RNA/DNA hybrids where primer extension cannot be performed.

Keywords: retroviruses; polypurine tract; RNA/DNA hybrid; chemical modification; mass spectrometry; NMR spectroscopy

INTRODUCTION

Plus strand, DNA-dependent DNA synthesis in retroviruses and long terminal repeat (LTR)-containing retrotransposons initiates from a purine-rich segment of the viral RNA genome designated the polypurine tract (PPT) (Klarmann et al. 2002; Rausch and Le Grice 2004). Subsequent PPT removal from nascent DNA is critical to creating the appropriate 5' LTR terminus for recognition by the viral integration machinery. While the accuracy with which the primer terminus is created from the RNA/DNA replication intermediate and the PPT is later excised from nascent

DNA can be accurately reproduced *in vitro* (Rausch and Le Grice 1997), the structural basis for its selection remains unclear. Recent single-molecule studies have indicated that dynamic binding orientations may direct the DNA polymerase and ribonuclease H (RNase H) activities of human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT), i.e., the composition of the PPT primer dictates whether enzyme favors its polymerization- or RNase H-competent state (Abbondanzieri et al. 2008).

The possibility that such complex protein/nucleic acid interplay might exploit unusual structural features of the duplex has been suggested by crystallographic analysis of HIV-1 RT bound to a PPT-containing RNA/DNA hybrid (Sarafianos et al. 2001). This study highlighted a collection of weakened base pairs, unpaired and mispaired bases, collectively designated the “unzipped” region (Fig. 1). However, whether these structural rearrangements result from enzyme binding or reflect an unusual, pre-existing geometry exploited by RT remains to be established. To address

Reprint requests to: Stuart F.J. Le Grice, RT Biochemistry Section, HIV Drug Resistance Program, National Cancer Institute, National Institutes of Health, Frederick, MD 21702, USA; e-mail: legrices@mail.nih.gov; fax: (301) 846-6013.

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Selective 2'-hydroxyl acylation analyzed by mass spectrometry (SHAMS)

An important concern in chemical probing of nucleic acids is the possibility that initial modification of exposed sites induces conformational changes of the target structure, exposing additional sites that were originally protected. The resulting modification pattern would therefore not provide an accurate representation of the native structure, but include significant unwarranted deviations. Subtle variations of pH and ionic strength associated with the probing reaction can also contribute to distorting the substrate conformation, potentially leading to erroneous and artifactual information. These concerns can be minimized by performing chemical probing under “single-hit” conditions by judiciously selecting the probe:substrate ratio. Unfortunately, the leading analytical platforms make verifying fulfillment of single-hit conditions problematic. Primer extension, for example, relies on the ability of NMIA modification to halt DNA polymerization by reverse transcriptase, thus producing oligonucleotides of specific length determined by the adduct position. However, when the primers are targeted only to the substrate ends, polymerization will effectively stop at the first adduct encountered, while any modifications existing downstream on the same molecule will remain undetected.

Chemical probes can be employed with direct mass spectrometric detection, due to unique mass signatures that enable immediate characterization of their stoichiometry and sequence position (Yu and Fabris 2003, 2004; Kellersberger et al. 2004). The flexibility afforded by this platform allows utilization of both mono- and bifunctional probes, which are critical for revealing long-range interactions and spatial relationships necessary to model complex three-dimensional structures (Yu et al. 2005, 2008). More significantly, the ability to directly monitor the number of modified sites provides the basis for an effective strategy for recognizing the occurrence of probing distortion, which consists of titrating the target structure with increasing amounts of probe in individual reactions. In this way, the onset of possible distortion and unfolding can be readily recognized by the detection of new sites that become reactive in the titration series.

This approach is exemplified by NMIA probing of the PPT RNA/DNA hybrid and direct monitoring by ESI-FTICR. As shown by the spectrum obtained in a 10-fold NMIA excess, up to three NMIA adducts are clearly recognizable from their characteristic incremental mass of 133.05 Da, together with a significant proportion of unmodified RNA (Fig. 2A). The complementary DNA strand is also observed, due to melting of the RNA/DNA hybrid before MS analysis. All species display the sodium adducts typically observed in nucleic acid analysis (Turner et al. 2008b). As the NMIA concentration was progressively increased, adduct intensity increased and that of unmod-

FIGURE 2. Nano-ESI mass spectra of HIV-1 PPT hybrid after reaction with a (A) 10-fold, (B) 50-fold, and (C) 100-fold NMIA excess for 50 min at 37°C. Under minimal reaction conditions, the majority of the RNA substrate remains unmodified, while in the *bottom* spectrum the entire RNA substrate is modified. In both cases, the DNA strand is not modified by NMIA owing to the absence of a ribose 2'-OH group.

ified RNA decreased correspondingly (data not shown). However, new adducts were only noted at a 50-fold NMIA excess, concomitant with which was the loss of all unmodified substrate (Fig. 2B). The number of adducts increased linearly with probe concentration, to the point where all possible 2'-OH groups were found to be susceptible to modification by a 100-fold of reagent (Fig. 2C). Product accumulation observed at higher NMIA concentrations, followed by the sudden increase of the number of adducts, suggesting substrate unfolding induced by modification of primary sites in the native hybrid.

Mass spectrometry can, in addition, provide direct information about the relative abundance of the modification products, which can be problematic for other analytical platforms. Indeed, the negative charges necessary for ESI-FTICR detection are carried by the phosphate groups of the oligonucleotide backbone, which are unaffected by ribose modification. This observation is substantiated here by the fact that all RNA species in Figure 2 (modified and unmodified) exhibited the same charge state. Therefore, signal intensities were a direct reflection of the respective abundances in solution, providing a relative scale of reactivity at different sites.

NMIA sensitivity of HIV-1 PPT ribonucleotides

Confident that the greater reactivity directly reflected structural flexibility, rather than conformational effects induced by initial modifications, we next proceeded to map the position of the sites exposed at the lower NMIA

readily recognized (Fig. 3A), as typically expected from this type of nucleic acid molecule (Cerny et al. 1987; Kirpekar and Krogh 2001; Kellersberger et al. 2004; Tromp and Schurch 2005). Activation of the triply modified species enabled detection of c ions that were shifted by the characteristic NMIA incremental mass (Fig. 3B), thus revealing the sequence position of the various adducts (Fig. 3B, inset).

nucleotides reflects fraying of the duplex ends, consistent with our recent NMR studies (Yi-Brunozzi et al. 2008). Acylation of ribonucleotides -12a and -11g (Fig. 3C) may be a direct consequence of their mispairing with template nucleotides -13T and -12T, respectively, determined from the co-crystal structure of HIV-1 RT bound to its PPT (Sarafianos et al. 2001). In contrast, NMIA reactivity provided no evidence for the unpairing of ribonucleotide -13a, as suggested by the crystal structure. In this respect, it is important to note that while KMnO_4 probing indicated accessibility of template thymines -15T, -14T, -13T, and -12T, suggesting deviation from canonical Watson-Crick geometry (Kvaratskhelia et al. 2002), no such anomalies were reported by the NMR analysis that instead confirmed Watson-Crick base-pairing throughout the HIV-1 PPT RNA/DNA hybrid in the absence of RT (Yi-Brunozzi et al. 2008). In particular, no upfield shifting of imino protons was observed, as might be expected based on the -11g/-12T mismatch observed in the crystal structure of Sarafianos et al. (2001).

A

c₁ a₂ a₃ a₄ a₅ g₆ a₇ a₈ a₉ a₁₀ g₁₁ g₁₂ g₁₃ g₁₄ g₁₅ g₁₆ a₁₇ c₁₈ u₁₉ g₂₀

Relative Intensity

c₁ y₂ y₄ c₂ c₄ y₅ c₅ c₈ y₅ c₃ c₆ c₉ c₁₀ y₇ c₇ y₈ [HIV_{RNA}-5H]⁺

B

c₁ a₂ a₃ a₄ a₅ g₆ a₇ a₈ a₉ a₁₀ g₁₁ g₁₂ g₁₃ g₁₄ g₁₅ g₁₆ a₁₇ c₁₈ u₁₉ g₂₀

Relative Intensity

c₁⁺ y₂⁺ c₂⁺ y₄⁺ c₄⁺ y₅⁺ c₅⁺ y₆⁺ c₆⁺ y₇⁺ c₈⁺ y₈⁺ c₉⁺ y₁₀⁺ c₁₀⁺ y₁₁⁺ c₁₁⁺ y₁₂⁺ c₁₃⁺ y₁₄⁺ c₁₅⁺ y₁₆⁺ c₁₇⁺ y₁₈⁺ c₁₉⁺ y₂₀⁺ [HIV_{RNA}+3NMIA-5H]⁺

C

3' (G) (T) (T) (T) (T) (C) (T) (T) (T) (T) (C) (C) (C) (C) (C) (C) (T) (G) (A) (C) 5'

5' (C) (a) (a) (a) (a) (a) (a) (a) (a) (a) (g) (g) (g) (g) (g) (g) (a) (c) (u) (g) 3'

-15 -10 -5 -1 +1

PPT ⇐ U3

1608 RNA, Vol. 15, No. 8

transient loss of base-pairing (Kopka et al. 2003). Alternatively, $-12a/-11g$ NMIA sensitivity may reflect an alteration in sugar pucker from the C3'-endo to C2'-endo conformation, which has been suggested to mediate ligand recognition (which in this case would be HIV RT) and catalysis (Vicens et al. 2007; Gherghe et al. 2008).

NMIA sensitivity of analog-substituted HIV-1 PPTs

Previous studies have shown that introducing the non-hydrogen-bonding thymine isostere 2,4-difluoro-5-methylbenzene (dF) at selected positions of the DNA template in the HIV-1 PPT facilitates NMR assignment of many carbon and proton resonances of the PPT substrates while also acting as a subtle probe of hybrid structure (Yi-Brunozzi et al. 2008). dF substitutions within the PPT DNA were also shown to significantly re-direct RNase H cleavage specificity of the modified RNA/DNA hybrids (Rausch et al. 2003). Based on these combined observations, dF was substituted for $-13T$, $-8T$, and $+1T$ on the PPT DNA template, and NMIA sensitivity of analog-substituted RNA/DNA hybrids was examined (Fig. 4).

A $-13T/dF$ substitution resulted in a maximum of four NMIA adducts under the same reaction conditions employed for the wild-type hybrid, which SORI-CID revealed as being produced by the modification of five distinct sites (data not shown). As observed for the wild-type hybrid, terminal ribonucleotides $-16c$ and $+4g$, along with internal ribonucleotides $-12a$ and $-11g$, were acylated (Fig. 4A). Modification of the additional site corresponding to ribonucleotide $-13a$ was directly ascribable to elimination of

hydrogen-bonding capacity with dF (Fig. 4A). In contrast, a $-8T/dF$ substitution eliminated acylation of PPT ribonucleotides $-11g$ and $-12a$ (Fig. 4B). Equally unexpected was the finding that ribonucleotide $-8a$ remained insensitive to acylation despite its inability to pair with $-8dF$. Finally, a hybrid wherein dF was substituted for $+1T$ retained reactivity at the internal ribonucleotides $-12a$ and $-11g$, but failed to elicit NMIA sensitivity of the $+1a$ RNA complement, analogous to our observations with the $-8T/dF$ template substitution (Fig. 4C).

Given the unusual acylation patterns of dF-substituted hybrids, ^{19}F -NMR was next used to determine if anomalous structural features could be detected at the sites of analog replacement. What is immediately apparent from the 1D ^{19}F spectra of the $-13T/dF$ and $-8T/dF$ -substituted templates (Fig. 5A,B) is that the modified PPT hybrids exhibited two major and at least two minor peaks for the fluorine resonances associated with the dF substitution. Since the purity of the substituted DNA strand and RNA:DNA stand stoichiometry were confirmed by mass spectrometry, the presence of the minor peaks suggests a small population of an alternative conformer(s). In addition, while the pyrimidine isostere exists in a 5'-T-T-dF-T-C-3' sequence context for both the $-13T/dF$ - and $-8T/dF$ -substituted templates, the chemical shifts associated with the fluorine resonances of the dF bases are significantly different, indicating differences in the local stacking of these bases in the RNA/DNA hybrid (Fig. 5A,B). Moreover, NOE correlation patterns observed in HF NOESY experiments acquired on the $-13T/dF$ - and $-8T/dF$ -substituted templates (data not shown) are consistent with minor

groove compression in the central A-tract. These distinguishing observations of altered base-stacking and minor groove compression presumably renders the 2'-hydroxyl inaccessible to acylation with NMIA at position -8 despite the loss of hydrogen-bonding capacity. Furthermore, the ability to alter the acylation profile at a distance through a relatively benign insertion of a thymine isostere indicates an extraordinary sensitivity of the "unzipped" region of the PPT RNA/DNA hybrid to minor conformational changes.

The 1D ^{19}F spectrum of the $+1T/dF$ -substituted RNA/DNA hybrid differs from that of its $-8T/dF$ - and $-13T/dF$ -substituted counterparts (Fig. 5C) in that no minor peaks are detectable. This indicates a unique environment at the PPT/U3 junction with even greater stacking rigidity at the $+1a$ site that prevents access of NMIA (analogous to our observations with the $-8T/dF$

FIGURE 4. Schematic of the HIV-1 PPT RNA/DNA hybrid with (open diamond) T/dF substitutions at template positions (A) -12 , (B) -8 , and (C) $+1$. Gray pentagons indicate positions of NMIA sensitivity observed by mass spectrometry.

FIGURE 5. 1D ^{19}F NMR spectra of the HIV-1 PPT RNA/DNA hybrid with T/dF substitutions at template positions (A) -12 , (B) -8 , and (C) $+1$. Spectra were acquired at 30°C with the fluorine carrier frequency set to -118 ppm, sweep widths of 5646 Hz, 1024 scans, and 4K complex points. The F2 and F4 resonances for each dF base are labeled; asterisks indicate minor conformations.

hybrid), despite loss of hydrogen-bonding capacity. While the ^{19}F spectrum and lack of acylation at $+1\text{a}$ in the $+1\text{dF}$ -substituted hybrid suggests rigidity at this position, the PPT/U3 junction is selectively targeted by the aminoglycoside neomycin B, which has been shown to interact with

motifs that disrupt regular double-helical patterns (Turner et al. 2008a). Similarly, chemical footprinting indicated that template $+1\text{T}$ of the wild-type PPT RNA/DNA hybrid is sensitive to KMnO_4 oxidation, suggesting that the duplex structure at the PPT/U3 junction deviates from canonical Watson–Crick geometry.

NMIA sensitivity of the Ty3 retrotransposon PPT RNA/DNA hybrid

Ty3 is a *S. cerevisiae* long terminal repeat–containing retrotransposon whose reverse transcription process shares many features with HIV-1. However, while Ty3 plus-strand DNA synthesis initiates from an RNA primer whose sequence, $5'\text{-gagagaggaa-3'}$, differs significantly from the $(\text{rA:dT})_n:(\text{rC:dG})_n$ architecture of retroviral PPTs (Rausch and Le Grice 2004), the Ty3 PPT is accurately processed, extended, and removed from nascent DNA by its cognate RT (Rausch et al. 2000). Since SHAMS highlighted structural anomalies in the wild-type HIV-1 PPT that were not detected by NMR (Yi-Brunozzi et al. 2008), the Ty3 PPT was investigated to determine if similar anomalies existed and if they could be modified by altering helix architecture.

A maximum of three NMIA adducts were observed on the wild-type Ty3 PPT RNA (data not shown). Gas-phase activation of the triply modified substrate indicated the terminal ribonucleotides (-19a , $+5\text{u}$, and $+6\text{u}$) were NMIA-sensitive, as well as the ribose of $+1\text{g}$ (Fig. 6A). While modification of the terminal bases is attributed to fraying at the hybrid termini, selective modification of $+1\text{g}$ is quite striking and consistent with previous NMR analysis that has shown an equilibrium between C3'-endo and C2'-endo sugar puckers at this position that may reflect conformational malleability at the PPT/U3 junction (Yi-Brunozzi et al. 2005).

Surprisingly, introducing a g:T wobble pair at position -6 renders the ribose of -5a , -6g , and -7a NMIA-sensitive,

FIGURE 6. (A) Schematic of the wild-type *S. cerevisiae* Ty3 PPT RNA/DNA hybrid and (B) a variant containing a -6G:T mismatch. Filled pentagons indicate NMIA sensitivity observed by mass spectrometry. The -6G:T mismatch generates local sensitivity to NMIA at positions -7 , -6 , and -5 , but also eliminates sensitivity at (*) position $+1$.

while negating reactivity at position +1g (Fig. 6B). Our observations are quite striking as they show that this type of base-pair substitution, where pairing is presumably maintained, can significantly affect duplex conformation distal to the site; i.e., it is not restricted to adjacent backbone sugars. Thus, both the HIV-1 and Ty3 PPT hybrids appear to exhibit the potential for long-range structural coupling that might be exploited by their cognate RTs.

DISCUSSION

Our study of the HIV-1 and Ty3 PPTs clearly demonstrates that combining NMIA acylation with mass spectrometric characterization allows probing of short RNAs on which the primer extension step of SHAPE is impractical, or of substrates whose sequence or secondary structure prevents efficient primer extension. In support of these latter considerations, we note that, even in the absence of chemical modification, the PPT (rG)₆ tract poses a significant barrier to reverse transcription of the RNA strand, resulting in prematurely terminated cDNA (JW Rausch and SFJ Le Grice, unpubl.). A particular advantage of the SHAMS method described here is that the ability to directly monitor the number and position of modified sites permits titration approaches to examine conformational effects induced by primary chemical modification. Based on these characteristics and the broad applicability of its analytical platform, the SHAMS approach should find general utility in structural biology. Our results obtained from PPT-containing hybrids substituted with the thymine isostere dF also provide a note of caution in interpreting the results of NMIA reactivity, which cannot be always linked solely to the absence of base-pairing. Conversely, the lack of 2'-OH modification should not be ascribed only to nucleotide participation in base-paired regions, but also to other effects constraining the ribose pucker conformation and to the steric protection induced by noncanonical base-pairing, unusual base-stacking, cognate proteins, or other ligands, which is always possible with *in vivo* applications.

While NMR analysis suggests Watson-Crick base-pairing throughout the HIV-1 PPT in the absence of RT, with no evidence for unpaired or mispaired bases (Yi-Brunozzi et al. 2008), two acylation-sensitive internal positions (−11g and −12a) were detected by the SHAMS strategy. These new data are consistent with previous footprinting studies indicating that template thymine −11T and −12T are modestly responsive to KMnO₄ oxidation in the absence of the retroviral polymerase (Kvaratskhelia et al. 2002). In both footprinting strategies, the reactive species represented a small fraction of the total population. Considering the results from the footprinting experiments, as well as the NMR data and crystal structure of the HIV-1 PPT•RT complex (Sarafianos et al. 2001), we propose that malleability of the HIV-1 PPT sequences around the 5'-aga-3' step between positions −12 and −10 is exploited

to accommodate HIV-1 RT in a manner such that the active site of its C-terminal RNase H domain is correctly positioned over the scissile phosphodiester bond at the PPT-U3 junction (Fig. 1). It is interesting to note that, in this orientation, the malleable region would be immediately downstream from the DNA polymerase domain where crystallography indicates that the duplex is bent by ~40° (Jacobo-Molina et al. 1993; Huang et al. 1998; Sarafianos et al. 2001). Such malleability may manifest itself in transient unpairing or altered sugar pucker, thus rendering the ribose 2'-OH NMIA accessible. Sensitivity of this region to minor alterations in the local environment is exemplified by loss of acylation when template base −8T is replaced with dF (Fig. 4B). In addition, our recent analysis of RT/PPT complexes by single-molecule spectroscopy has shown that minor alterations to the PPT can reverse enzyme orientation at the primer terminus (Abbondanzieri et al. 2008).

In summary, we demonstrate that chemical acylation can be directly applied to probe conformational features of the RNA of RNA/DNA hybrids, with emphasis here on the plus-strand, PPT RNA primers of a human retrovirus (HIV-1) and *S. cerevisiae* LTR-containing retrotransposon (Ty3). Although not widespread, there are several examples of RNA/DNA hybrids with biological significance, including their involvement in immunoglobulin class switching (Tracy et al. 2000), human cytomegalovirus virus lytic DNA replication (Pari 2008), and ColE1 plasmid replication (Santamaria et al. 1998). The ease with which NMIA treatment can be combined with mass spectrometry should allow a more detailed analysis of these duplexes, either alone or in conjunction with their protein partners. Finally, while our SHAMS approach has broad utility in detecting subtle alterations in nucleic acid geometry, the HIV PPT represents a system in which interpreting these changes benefits from the availability of X-ray crystal structures, high-resolution NMR analysis, and biochemical probing of analog-substituted RNA/DNA hybrids. Complementing chemical probing with data obtained using other biochemical and biophysical techniques will clearly be required to interpret the biological significance of such subtle structural modifications in other biological systems.

MATERIALS AND METHODS

Nucleic acid substrates

RNA and DNA phosphoramidites, including 2,4-difluoro-5-methylbenzene, were purchased from Glen Research Corp. Oligonucleotides were synthesized at a 1-μmol scale using standard phosphoramidite chemistry on a PE Biosystems Expedite 8909 nucleic acid synthesizer and purified by preparative polyacrylamide gel electrophoresis. RNA and DNA single strands were annealed by mixing equimolar amounts, heating the solution for 3 min to 90°C, and allowing the samples to cool slowly to form the

duplex. Duplex concentrations were determined from absorbance at 260 nm (CARY300 UV-Vis Spectrophotometer), using a molar extinction coefficient that is equal to the sum of the ribo- and deoxyribonucleotides ($384,900\text{ cm}^{-1}\text{ M}^{-1}$ and $379,700\text{ cm}^{-1}\text{ M}^{-1}$ for Ty3 and HIV-1 PPT duplexes, respectively). Final duplex concentrations for NMR measurements were typically 1.0 mM.

Nucleic acid substrates

Through titration and time-course experiments, chemical acylation reactions were optimized to avoid possible substrate distortion, as described in the text. As a result, the data discussed in Results and Discussion required reacting 50 μM RNA/DNA hybrid with up to 500 μM NMIA (final solution concentrations) for 50 min at 37°C. Increasing the final probe concentration to greater than 10-fold excess induced multiple nonspecific probing events.

Mass spectrometry

MS analyses were performed on a Bruker Daltonics Apex III FTICR mass spectrometer equipped with a 7T actively shielded superconducting magnet and a nano-ESI source constructed in house. Reaction mixtures were quenched via ethanol precipitation. The pellet was resuspended in 5 mM ammonium acetate and warmed to 80°C to reduce the hybridization and allow for more facile data analysis. Typically, 5 μL of the warm analyte solutions was loaded into the nano-ESI needle, and a spray voltage of <1 kV was applied to the solution through a stainless steel wire inserted in the rear end of the needle. No solvent pumps were necessary, as the solution flow rate was dictated by the applied voltage and size of the nano-ESI needle tip (typically $\sim 1\text{--}2\text{ }\mu\text{m}$). Spectra were acquired in negative ionization mode and processed using ApexControl 2.0 (Bruker Daltonics). Scans were completed in broadband mode that allowed for a typical 150,000 resolving power at m/z 2000. Spectra were externally calibrated using a 1 mg/mL solution of CsI, which produced a series of peaks throughout the mass range of 1000–6000 m/z and provided a typical mass accuracy of 20 ppm or better across the range. Each analysis was performed a minimum of three times.

In tandem MS experiments, the precursor ion of interest was isolated in the FTICR cell using correlated rf sweeps (de Koning et al. 1997), then activated through SORI-CID (Gauthier et al. 1991). Frequency offsets below and above the resonant frequency of the precursor ion were sampled to avoid possible “blind spots” in the product spectra. Best results were achieved with irradiation frequencies 600 to 2000 Hz below that of the precursor ion. Typical activation regimes were reached by applying off-resonant pulses for 250 msec, using 26–31 dB attenuation of the maximum power output allowed by the hardware. Argon was used as the collision gas in pulsed bursts of 100–250 msec, which resulted in momentary increases from 1×10^{-11} to 1×10^{-7} mbar of the background pressure measured by the instrument gauge located underneath the ion optics. No attempt was made to determine the actual pressure within the FTICR cell.

NMR analysis

NMR samples were examined in 80 mM NaCl and 10 mM phosphate (pH 7.0) that were dissolved in 90% H_2O /10% D_2O or

99.96% D_2O . One-dimensional ^{19}F experiments were performed on a Bruker Biospin DRX500 spectrometer equipped with a direct-detect ^{19}F probe. Two-dimensional spectra were collected on a Bruker Biospin DMX600 equipped with either a TXI HCN probe or a PH SEF 600SB F-H-D-05 Z probe. All spectra were processed with Topspin and analyzed using Sparky or Topspin on a PC/Linux workstation. Assignments were made using DQF-COSY, NOESY, TOCSY, $^1\text{H}\text{--}^{13}\text{C}$ (natural abundance) HMQC, $^1\text{H}\text{--}^{19}\text{F}$ HSQC, and $^1\text{H}\text{--}^{19}\text{F}$ NOESY. Other experimental details and acquisition parameters are given in the Figure 5 legend.

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