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Practical Considerations and Guidelines for Spectral Referencing for Fluorine NMR Ligand Screening

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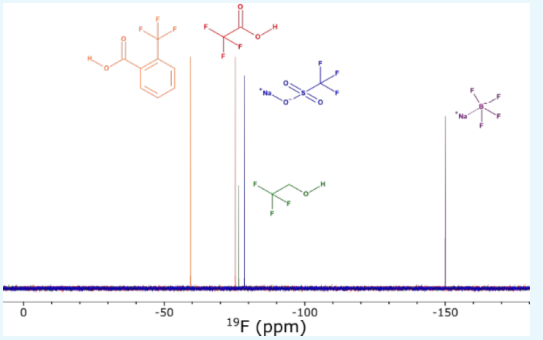
ABSTRACT: Fluorine (^{19}F) NMR strategies are increasingly being employed for evaluating ligand binding to macromolecules, among many other uses. ^{19}F NMR offers many advantages as a result of its sensitive spin 1/2 nucleus, 100% natural abundance, and wide chemical shift range. Moreover, because of its absence from biological samples, one can directly monitor ligand binding without background interference from the macromolecule. Therefore, all these aforementioned features make it an attractive approach for screening compounds. However, the detection of ligand binding, especially those with weak affinities, can require interpretations of minor changes in chemical shifts. Thus, chemical shift referencing is critical for accurate measurements and interpretations. Unfortunately, one cannot rely on spectrometer indirect referencing alone, and internal chemical references have sample-dependent issues. Here, we evaluated 10 potential candidate compounds that could serve as ^{19}F NMR chemical references. Multiple factors were systematically evaluated for each candidate to monitor the suitability for ^{19}F NMR screening purposes. These factors include aqueous solubility, buffer compatibility, salt compatibility, aqueous stability, tolerability to pH changes, temperature changes, and compound pooling. It was concluded that there was no ideal candidate, but five compounds had properties that met the screening requirements.



INTRODUCTION

Fragment-based lead discovery (FBLD) involves the screening of low-molecular-weight compounds to identify binders to essential disease target proteins or nucleic acids. Nuclear magnetic resonance (NMR) is a very useful tool for FBLD due to its ability to detect weak binding events in a label- and immobilization-free environment. Traditional ligand-detected experiments have mostly been performed using proton (^{1}H) NMR, but fluorine (^{19}F) experiments have increasingly gained in popularity in recent years. The large, background-free chemical shift dispersion of the fluorine moiety, combined with its 100% natural abundance and high sensitivity to molecular interactions, has made it an attractive tool in the field of drug discovery.[1−17]

Binding events are usually detected by monitoring changes in chemical shifts, signal width, and/or peak intensities. Variation in the chemical shift is expected to occur if a difference between the bound and free states is experienced by the ^{19}F nucleus.[18] However, these observations can be skewed, as minor but significant changes in chemical shifts are common due to the wide spectral dispersion of the ^{19}F nucleus coupled with spectrometer instabilities and sample-dependent shift changes. Thus, one cannot always rely on spectrometer indirect referencing alone.

In general, the IUPAC recommendations favor internal referencing or substitution methods,[19] with internal referencing being generally more practical for a drug screening context. The IUPAC also recommends the use of CCl_3F as a reference compound for ^{19}F NMR, but this molecule presents several practical limitations: it has limited aqueous solubility, is highly volatile at ambient temperature, and possesses ozone-depleting properties, which restricts its commercial availability.[20] Analogously, currently recommended ^{19}F quantitative NMR references are intended to be used in organic solvents and are less optimal for screening, as their aqueous solubility is also limited.[21] Therefore, there are no definitive guidelines for ^{19}F NMR screening, and the choice of reference depends on lab-specific preferences or arbitrary reasons.[1]

Some essential characteristics should be considered in the choice of a ^{19}F NMR shift reference, keeping in mind that requirements can be project-dependent. These characteristics include solubility and stability in aqueous media, compatibility with common buffer components, absence of promiscuous binding to protein systems, chemical shift compatibility with standard NMR experimental screening parameters, and minimal chemical shift changes from variations in sample

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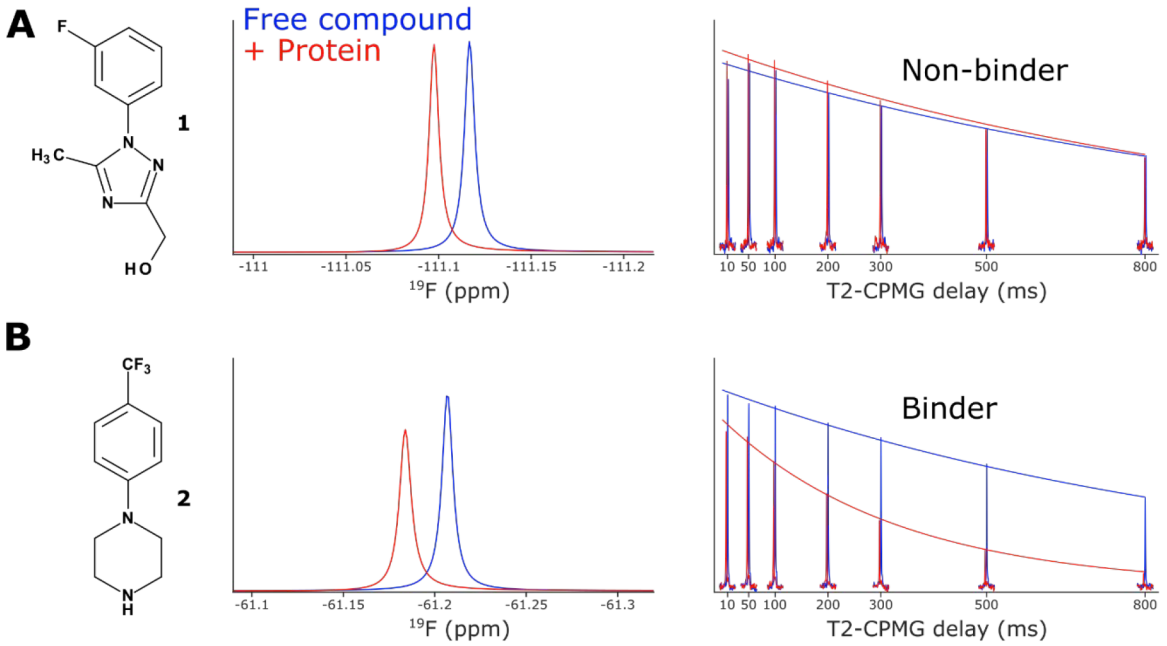

Figure 1. ^{19}F NMR screen using DLBS and T2-CPMG methods. (A) Compound **1** exhibits changes in chemical shift in the presence of protein (red spectrum), as compared to the free compound (blue spectrum). Further evaluation of the binding using T2-CPMG suggests that this compound is not a binder and that the chemical shift difference is likely explained by the sensitivity to small changes in conditions. In comparison, (B) shows compound **2**, which appears to be a real binder to the protein based on the significant differences in line broadening and T2-CPMG decay rate observed.

conditions (e.g., pH, temperature, dimethyl sulfoxide (DMSO) content, mixtures of compounds). Other desirable (but not essential) features include the presence of a polyfluoro moiety (e.g., CF_3), allowing for a sufficient ^{19}F NMR signal-to-noise ratio even at low concentrations of the reference compound in the sample to limit potential artifacts, as well as commercial availability, lack of safety concerns, and ease of handling. Furthermore, reference compounds that lack or have minimal nonexchangeable aromatic hydrogens would allow concurrent ^{1}H NMR experiments to be acquired on the same sample.

Herein, we evaluated some of the most commonly used fluorine shift references for ^{19}F NMR under a variety of conditions and environments to assess their suitability for drug discovery studies. We also provide some guidelines that may help users choose the most appropriate reference for their project.

RESULTS AND DISCUSSION

Example: Inconclusive Results in the Absence of an Internal Shift Reference. As with routine ^{1}H NMR applications, spectrometer indirect referencing is often preferred for ^{19}F NMR shift calibration. However, inconsistencies can arise from one sample to the next due to the reasons already described above.

One ramification is that interpretations can become ambiguous. An example is illustrated in Figure 1, which shows a ^{19}F NMR screen aimed at determining whether two compounds bind to a target protein. In panel A, the differential line broadening/shifting (DLBS) method would suggest that compound **1** could bind to the target protein given that the ^{19}F spectrum of free compound **1** (blue spectrum) experiences a distinct change in chemical shift upon the addition of target protein (red spectrum). However, a confirmational T2-CPMG (Carr−Purcell−Meiboom−Gill) experiment shows that compound **1** does not appear to bind the target protein.[22] On the one hand, no significant changes in relaxation rates (i.e., slopes as a function of delay periods) are observed between the samples containing free **1** (blue) and **1** with added protein (red). On the other hand, in panel B the changes in DLBS and T2-CPMG data (red vs blue spectra) support that compound **2** indeed binds to the target protein. Thus, perhaps the chemical shift changes in panel A were due to either spectrometer drift or local chemical environment changes rather than a binding event. The lack of a significant difference in the T2-CPMG spectra in the presence of protein supports this hypothesis; therefore, an analysis based on shift alone could result in the misinterpretation that compound **1** bound to the target protein. Hence, it is crucial that a ^{19}F chemical shift reference be employed in such a context.

Selection of ^{19}F Reference Compounds. A set of commonly used ^{19}F references was chosen for this study. Their names and abbreviations are as follows: **BTF**: benzotrifluoride; **DFB**: 1,2-difluorobenzene; **HFB**: hexafluorobenzene; **KF**: potassium fluoride; **$NaBF_4$**: sodium tetrafluoroborate; **PhF**: fluorobenzene; **TFA**: trifluoroacetic acid; **TFE**: trifluoroethanol; **TFMBA**: 2-(trifluoromethyl)benzoic acid; **Triflate**: sodium trifluoromethanesulfonate.

Solubility Tests. A chemical shift reference should exhibit sufficient solubility under aqueous conditions to be practical for NMR screening. The solubility of the 10 reference candidates was therefore assessed at 200 μM nominal concentration in 100% D_2O. Data were also acquired on samples at 200 μM nominal concentration in 100% DMSO-d_6 for comparisons of signal intensities. Figure 2 illustrates the respective signal intensities, as well as the overlaid spectra (far-right column), in D_2O and DMSO-d_6 for each molecule. PhF was completely insoluble in D_2O, while BTF, DFB, and HFB exhibited poor signal-to-noise in aqueous solution. These four candidates were therefore eliminated from further testing due

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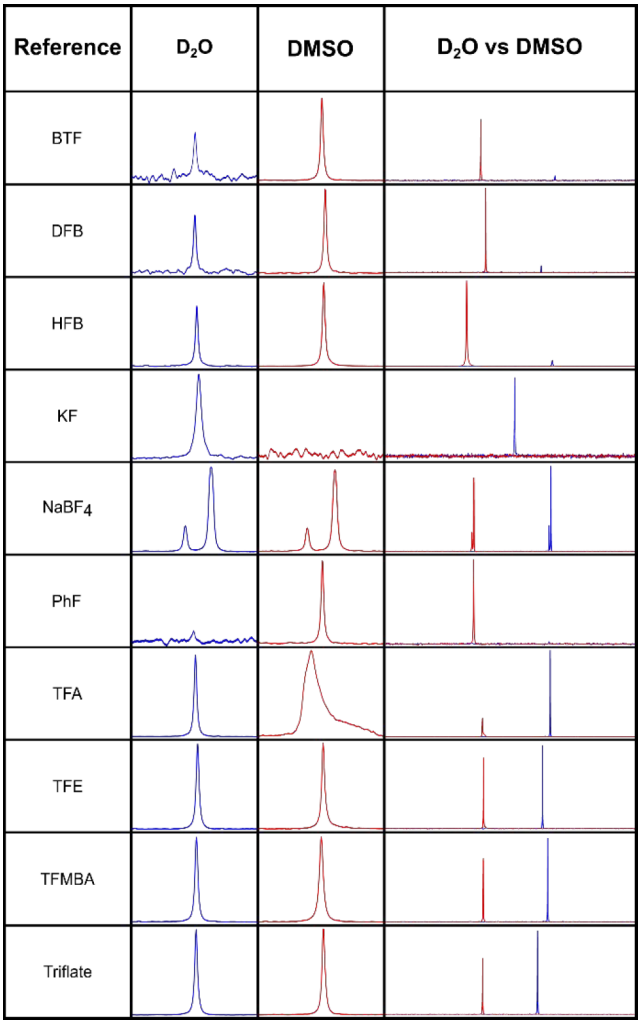

Figure 2. ^{19}F spectra of the candidate molecules were acquired in both D_2O (blue spectra) and DMSO-d_6 (red spectra) to evaluate their aqueous solubility. Overlaid spectra of both conditions (last column) allow for a better evaluation of the relative signal intensities. Compounds were tested at 200 μM nominal concentration.

to their insufficient solubility and, thus, incompatibility as a ^{19}F NMR screening reference. Even though it appeared to be insoluble in DMSO, KF showed sufficient signal-to-noise under aqueous conditions and was therefore advanced to further evaluations. Similarly, TFA showed a lower signal intensity in DMSO as well as a broader, nonsymmetrical peak line shape, which could perhaps be due to an exchange phenomenon. Given an acceptable intensity profile in D_2O, TFA was also considered for additional studies. Finally, $NaBF_4$, TFE, TFMBA, and Triflate all showed adequate solubility profiles and were also selected for the next rounds of testing.

Buffer Compatibility Tests. Another important attribute for a fluorine reference compound should be compatibility with various buffer conditions. The remaining six reference candidates were then evaluated for their compatibility with four common buffer components: sodium phosphate, potassium phosphate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and tris(hydroxymethyl)-aminomethane (tris). An analysis of the ^{19}F NMR spectra of the six candidates in these buffers is shown in Figure 3. Given the reasonable peak intensities in all conditions, it is apparent that all six are compatible with these buffers.

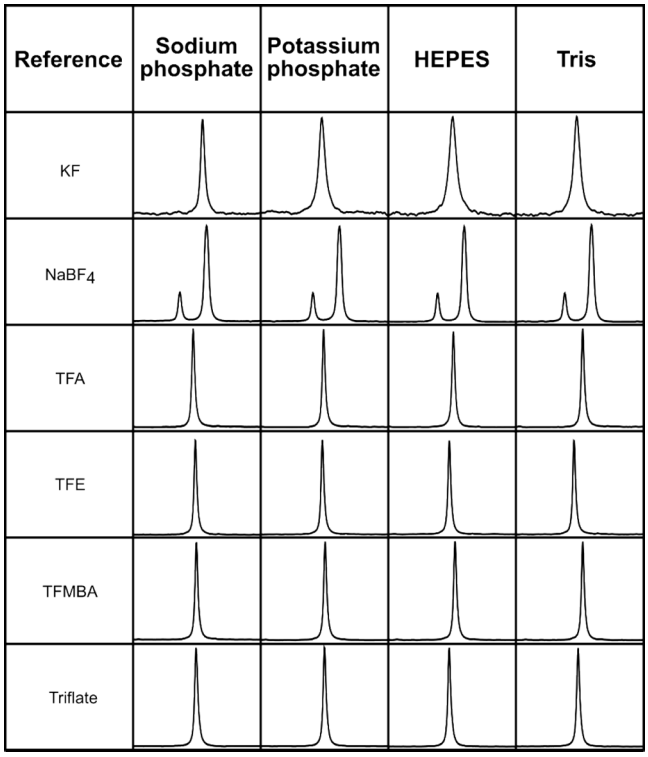

Figure 3. Compatibility of six candidates with common buffer components. 1D ^{19}F spectra are shown for compounds in each condition. Buffers were prepared at 50 mM, pH 7.0 with 10% D_2O, and compounds were tested at 200 μM nominal concentration.

Salt Compatibility Tests. For screening purposes, a fluorine reference should also be compatible with various salts. The compatibility with four commonly used salts (sodium chloride, potassium chloride, magnesium chloride, and calcium chloride) was then assessed as shown in Figure 4. With the exception of KF, all compounds were compatible with the four salts investigated. However, KF exhibited insolubility in the presence of magnesium chloride. This effect was further confirmed by titrating KF against a lower concentration (1 mM) of $MgCl_2$ (Figure S1), and significant loss of signal intensity and broad line widths could be observed, suggesting the formation of larger unknown entities in the sample. In a similar fashion, reduced signal intensity with a wider line shape was observed for KF in the presence of calcium chloride (Figure 4), which suggested limited compatibility with this salt as well. This is in line with the relatively limited solubility expected for MgF_2 and CaF_2.[23a,b] KF was therefore eliminated as a potential reference candidate. The remaining five molecules were then tested against a wide range of buffer components and additives (Figures S2−S5), and no significant incompatibility could be observed in any of the tested conditions.

Aqueous Stability Tests. Given that some NMR screening studies can last for several days, the aqueous stability of each remaining candidate was then assessed at three different time points: 0 h, 24 h, and one week. Figure 5A shows that signal intensities remain relatively stable across all time points for the five compounds. Similarly, minor variations in chemical shift of less than 1 Hz were noted for periods up to a week (Figure 5B).

pH, %DMSO, Temperature, and Compound Pooling Tests. We then evaluated the "sensitivity" of the compounds'

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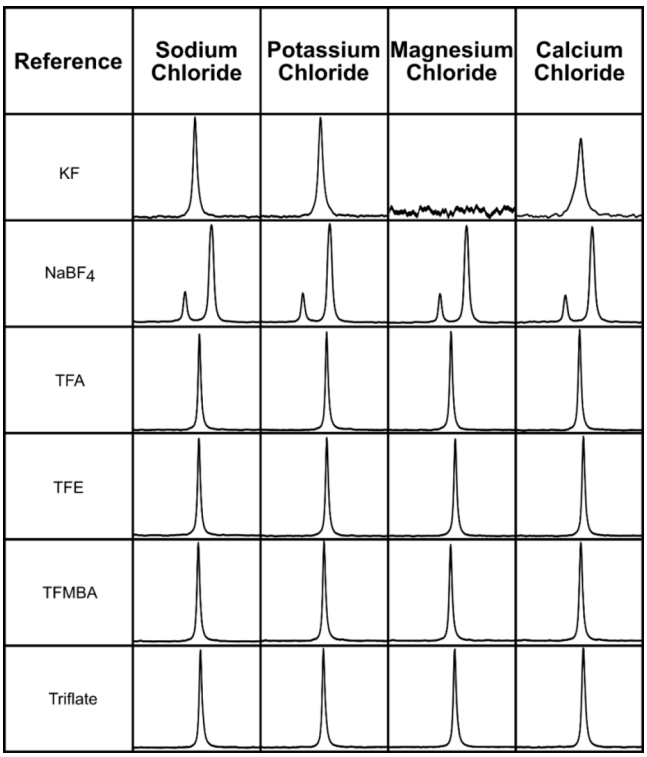

Figure 4. Compatibility of the six candidates with four common salts. 1D ^{19}F spectra are shown for compounds in each condition. Sodium and potassium chloride were tested at 200 mM, while magnesium and calcium chloride were tested at 50 mM. Compounds were tested at a nominal concentration of 200 μM, and solutions were prepared with 10% D_2O.

chemical shifts given variations in pH, amount of DMSO, temperature, and in the presence of pools of other compounds. Figure 6A shows that the chemical shift of $NaBF_4$ seems to be relatively sensitive to pH variations above pH values of 6 but that all the other molecules were rather stable across the pH range tested. Therefore, small variations in pH upon additions of various components, such as a protein or other compound, would only be expected to potentially cause more significant variations in the chemical shift of $NaBF_4$.

Similarly, because compounds are usually added into a buffer from DMSO solvent stocks, there was concern that chemical shifts of the potential reference compounds could be sensitive to different concentrations of DMSO (e.g., for drug titration purposes, from different stock concentrations, or even variations in pipetting). Thus, we were interested in evaluating if the reference candidates experienced changes in chemical shifts as the percentage of DMSO was altered. Figure 6B shows the changes in chemical shifts upon addition of 1, 2, 3, or 5% (v/v) DMSO-d_6. $NaBF_4$ was once again more influenced by variations in the amounts of DMSO.

Although temperature is usually well-controlled during NMR experiments, some NMR pulse sequences can result in some sample heating. Moreover, insufficient equilibration of the sample temperature before acquisition can also result in variation during the experiment. The influence of temperature fluctuation on the reference's chemical shifts was therefore probed by varying the temperature from 25 to 40 °C by 5 °C increments (Figure 6C). Most of the compounds show comparable changes in chemical shifts with varying temperature, but, interestingly, the chemical shift of $NaBF_4$ was the least affected by changes in temperature.

Fragment-based NMR screens are often performed using mixtures of compounds (pools) in order to increase throughput,[4,8,18,24−27] so an internal reference used in these screens should experience minimal effects on its chemical shift in the presence of other compounds. Each reference candidate was therefore tested in 20 different pools of small-molecule fragments containing 11−15 fragments per pool. Figure 6D shows the average change in chemical shift for each reference candidate when placed in the pools compared to the free compounds. It is noteworthy that $NaBF_4$'s chemical shift is, on average, more affected, while triflate's is the least affected. TFE, TFMA, and TFA exhibit comparable intermediate profiles. In light of the results observed in Figure 6A, the presence of multiple compounds in solution may induce slight changes in pH within the samples, and these changes could explain some of the observed effects. These results suggest that $NaBF_4$

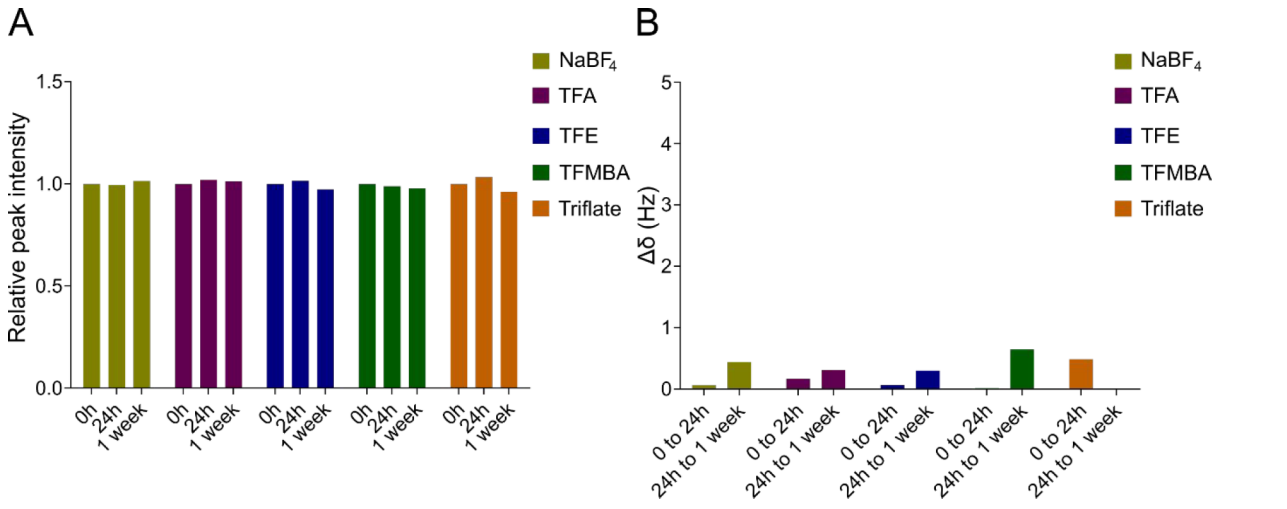

Figure 5. Evaluation of compound stability under aqueous conditions. 1D ^{19}F spectra were acquired at three time points: 0 h, 24 h, and one week. Signal intensities were measured at each time point (A) as well as variation in the ^{19}F chemical shift between each time point (B). Signal intensities in (A) were normalized to t = 0 h for each molecule. Samples were measured at 200 μM in 50 mM sodium phosphate pH 7.4, 100 mM NaCl, 10% D_2O.

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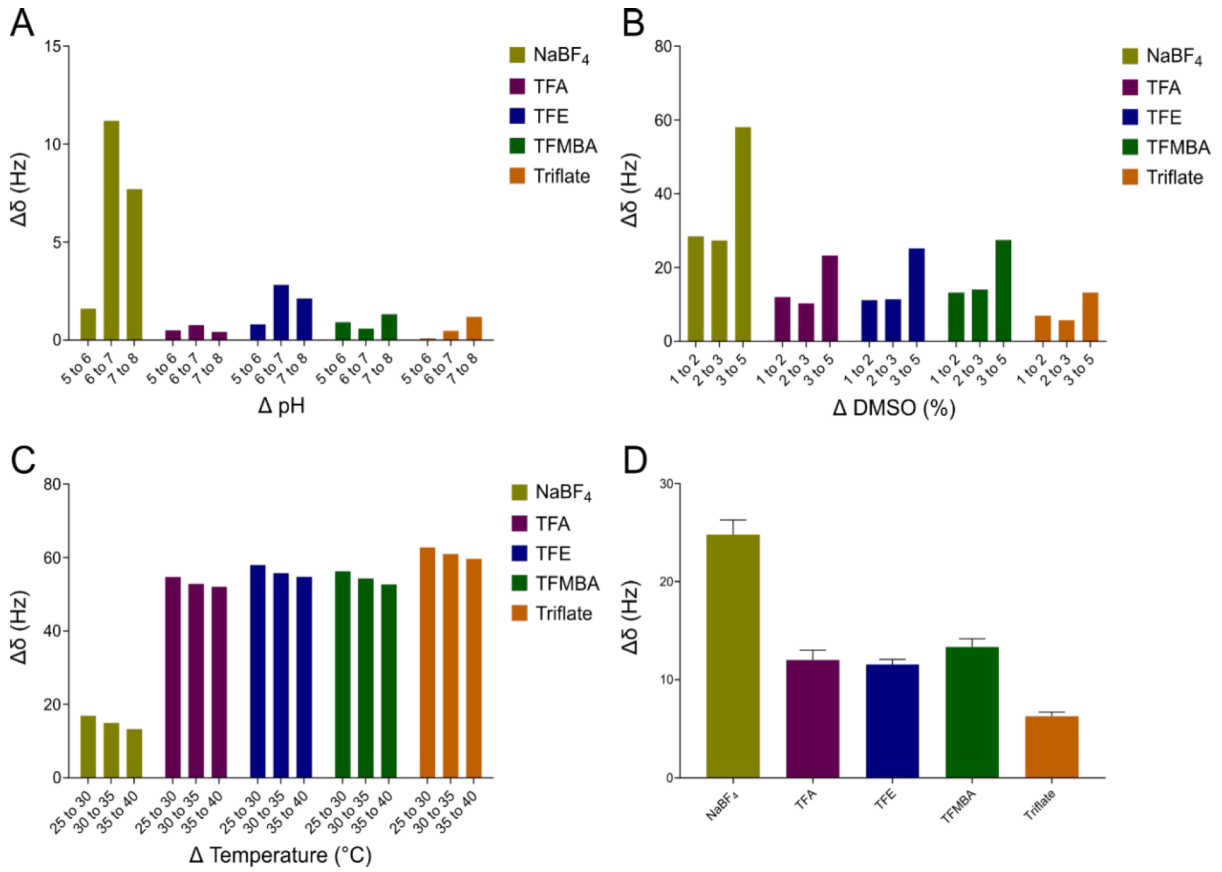

Figure 6. Fluorine chemical shift variations of five compounds upon variation of pH (A), DMSO-d_6 content (B), and temperature (C) and in the presence of pools of compounds (D). Samples for B and C were run in 50 mM sodium phosphate pH 7, 10% D_2O. For A, the same buffer was used as in B & C, at various pH values (5, 6, 7, and 8). In D, each molecule was tested against 20 pools of fragments for an average of ~13 fragments/pool. Average change in chemical shift is plotted with the standard deviation. Samples in D were run in 20 mM Tris-d_{11} pH 7.5, 150 mM NaCl, 10% D_2O.

would be a less-favorable option in the context of screening mixtures.

Ideally, an internal reference should not bind to the target macromolecule (usually a protein) that is being screened. Therefore, we screened the five remaining candidates against four different commercially available protein systems: bovine serum albumin (BSA), elastase, lysozyme, and trypsin. Binding was assessed at three different ligand-to-protein ratios (L/P) by monitoring changes in the ^{19}F differential line broadening (DLB) of each reference's signal upon addition of protein. Figure 7A shows that the addition of elastase, lysozyme, or trypsin results in little to no DLB effects under the various L/P tested. However, the addition of BSA results in relatively large DLB effects for TFA, triflate, and especially TFMBA (illustrated in Figure 7B) as the ligand-to-protein ratio approaches equimolar. Note that this is not unexpected considering that albumins are known to bind a wide variety of molecules.[28] However, this observation suggests that these reference candidates may be more prone to binding target macromolecules. Interestingly, $NaBF_4$ exhibited minimal DLB across all the conditions tested, suggesting that it might be less susceptible to protein binding.

Summary of the Main Pros and Cons for the Five ^{19}F NMR References. This study has shown that the evaluation of fluorine reference compounds for NMR screening must consider multiple parameters (Figure 8) and as a result is challenging. Overall, five reasonable candidate references emanated from this study. Table 1 summarizes the main pros and cons for each of these five references. Interestingly, all five contained a polyfluoro moiety (either a CF_3 group or BF_4^-) giving them sufficient signal-to-noise even if used at lower concentrations for referencing in NMR screens. Moreover, all five are commercially available and affordable.

Example Demonstration of the Utility of TFMBA for ^{19}F Chemical Shift Referencing in a Competition Study. Given the ensemble of data described herein, our laboratory frequently uses TFMBA as an internal reference for ^{19}F NMR screening studies. Figure 9 demonstrates an example of such a study for which the aim was to evaluate two compounds to determine whether they bound to a specific pocket (P-1) of the multipocketed target human rhinovirus polymerase (HRV). To do so, we had access to a known P-1 binder (^{19}F probe—see Figure 9B,C) that could serve as a displacement probe for other binders to pocket P-1. This probe also contained a CF_3 group, which increased the ^{19}F NMR signal-to-noise while also exhibiting relatively sharp linewidths due to a fast rotation along the CF_3–phenyl bond. Given this, samples were prepared with the internal TFMBA ^{19}F reference at a lower concentration of 5 μM. The low concentration of both the reference and the probe (^{19}F probe at 22 μM) would minimize any potential intermolecular association and interference. As a precaution, separate control experiments were run and showed that the TFMBA reference did not bind to the probe, HRV protein, or to the two test

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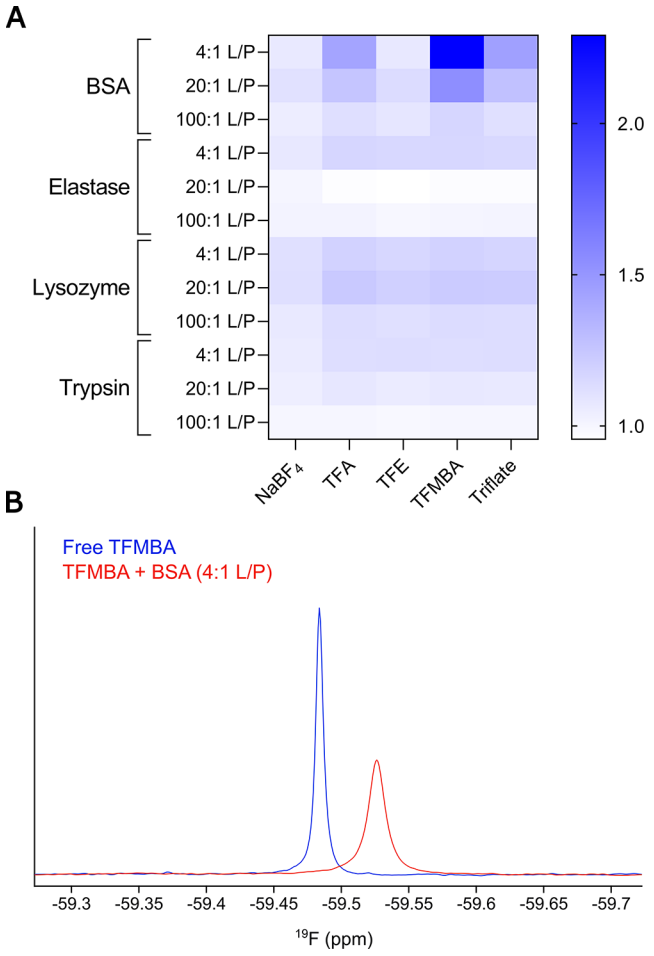

Figure 7. (A) Evaluation of binding to various proteins by fluorine DLB. Proteins were added at three different concentrations to 200 μM compound. Samples were tested in 50 mM sodium phosphate pH 7.0, 100 mM NaCl, 10% D_2O. Broadening was measured as a ratio of fluorine peak line width in the presence/absence of protein. A line broadening ratio of 1 represents the absence of any evidence of binding. (B) Example for TFMBA binding in the presence of BSA at 4:1 L/P.

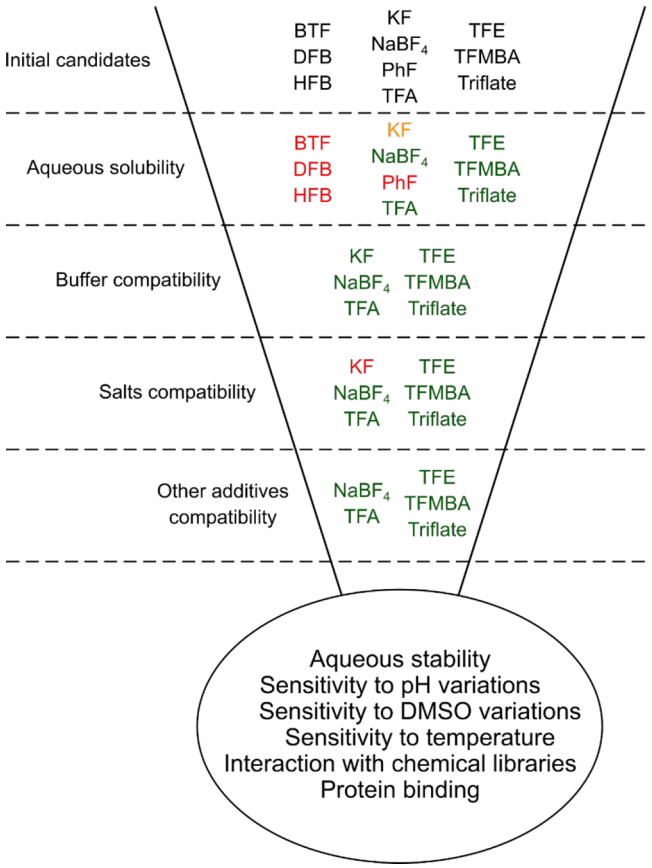

Figure 8. Funnel-like view of the reference evaluation steps. Compounds eliminated at each step are colored in red. KF was colored orange in the solubility assessment since it appeared soluble in aqueous conditions but insoluble in DMSO.

compounds (data not shown). Samples were then prepared with the three compounds present simultaneously (TFMBA at 5 μM, ^{19}F probe at 22 μM, test compound at 100 μM), both with and without HRV protein.

Key to the proper analyses of the data shown in Figure 9 was that all ^{19}F NMR spectra were chemical shift referenced to the TFMBA peak as shown in Figure 9A, which then facilitated accurate interpretations. Given that the ^{19}F probe and the test compounds were relatively weak binders, resonance averaging was expected due to fast exchange between the free and bound states (on the NMR time scale). Therefore, minor chemical shift changes would be expected upon binding. Upon addition of the HRV protein, the ^{19}F NMR resonance of the ^{19}F probe would shift downfield to that of the free ^{19}F probe, indicating a binding event. In the presence of a competitor test compound, however, as shown in the upper spectrum of Figure 9C, the resonance of the ^{19}F probe returned to its free-state chemical shift, indicating that it was competed out of pocket P-1 by the competitor test compound. In contrast, in the presence of a noncompetitor test compound, as shown in the upper spectrum of Figure 9B, the chemical shift of the ^{19}F probe remained the same as that of the sample without the noncompetitor compound. This result indicated that the ^{19}F probe remained bound, and the noncompetitor compound did not compete for pocket P-1. Without the chemical shift referencing to TFMBA, these results would have been much less convincing.

Additional Considerations. Several factors make identification of a universal ^{19}F internal reference very challenging. The main one being the large chemical shift distribution of ^{19}F compounds, which may require the use of a reference with a chemical shift close to the compound(s) of interest. For example, different references might be desired if mixtures are designed based on the presence of CF or CF_3, since they may require different carrier frequencies and spectral width depending on the experimental setup used.[4] Alternatively, the use of broadband ^{19}F pulse sequences can help circumvent this difficulty.

If users also plan to include ^{1}H NMR experiments for follow-up steps, then some ^{1}H-containing references will be less appropriate due to the potential for signal overlap with the compounds of interest. For example, Figure S6 shows the one-dimensional (1D) ^{1}H spectra of TFMBA and TFE. On the one hand, because of the absence of any aromatic protons on TFE, this mitigates potential problems of resonance overlap considering that aromatic protons are often favored during an NMR binding analysis due to the simplicity and the usual lack of any signal overlap with common buffer components. On the other hand, TFMBA possesses four aromatic protons,

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Table 1

reference compound	advantages	disadvantages
$NaBF_4$	Minimal chance of resonance overlapping with common fragments	Chemical shift might fall outside the spectral width of some NMR sequences
	Absence of ^1H	2 species observed due to natural abundance of boron isotopes
		Appears to be more sensitive to variations in DMSO or pH
TFA	Absence of ^1H	Can already be a residual from chemical synthesis
TFE	Only aliphatic ^1H	Volatile and flammable
		Aliphatic ^1H
		Known to promote protein changes at higher concentrations[29]
TFMBA	Decent all-around performance	Aromatic ^1H
		More fragment-like molecule, could be more prone to binding proteins
Triflate	Absence of ^1H	Appears to be slightly more sensitive to variations in temperature than other candidates
	Appears to be slightly less sensitive to variations in DMSO or pH than other candidates	

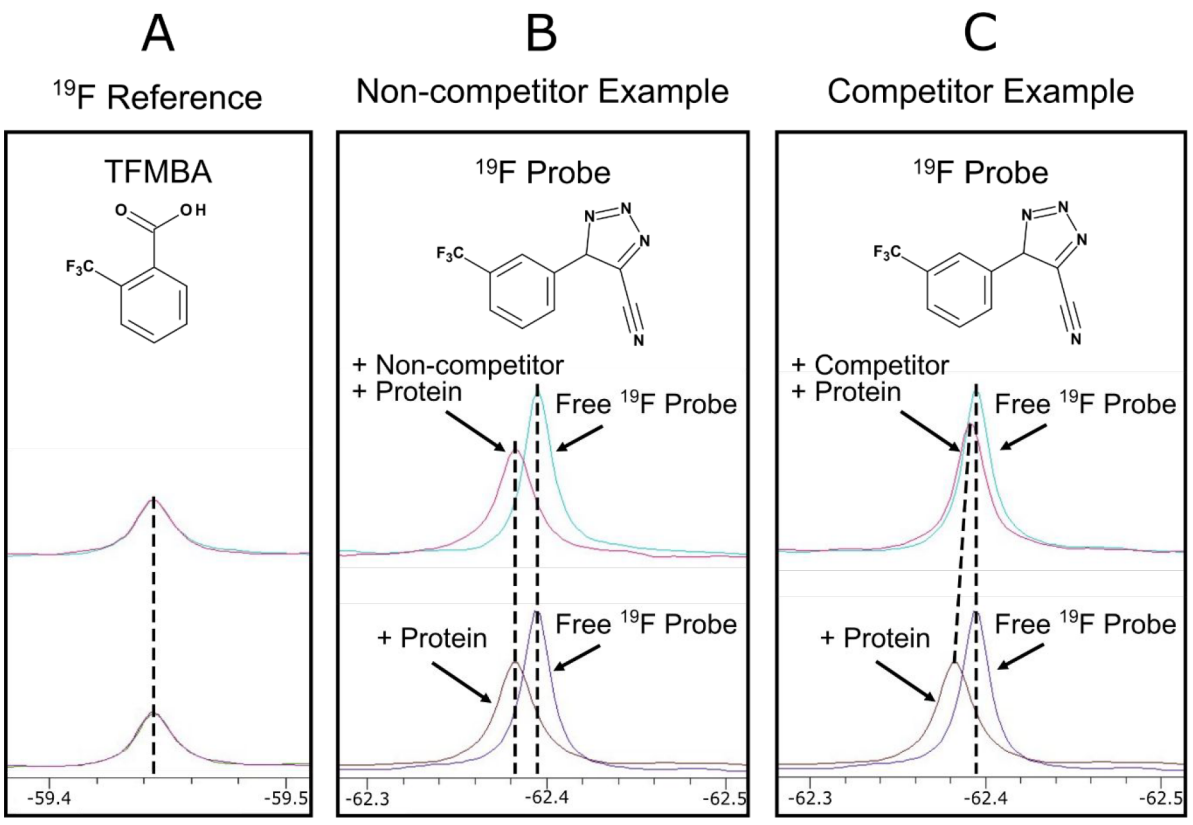

Figure 9. Demonstration of the utility of ^{19}F chemical shift referencing in a competition study. (A) ^{19}F NMR spectra of TFMBA (5 μM). (B) ^{19}F NMR spectra of ^{19}F probe at 22 μM, HRV at 22 μM, noncompetitor test compound at 100 μM. (C) ^{19}F NMR spectra of ^{19}F probe at 22 μM, HRV at 22 μM, competitor test compound at 100 μM.

which is likely to overlap various compounds of interest, as depicted in Figure S6B.

CONCLUSIONS

In summary, we have evaluated various fluorine reference candidates under various conditions to assess their suitability for NMR drug discovery experiments. We also highlighted the complexity of choosing an appropriate reference molecule and provided some recommendations to guide these choices. Evidently, there are likely other interesting reference candidates out there, and therefore, a similar testing sequence could be extended to other compounds to identify additional candidates. After a screen, a molecule defined as nonbinder can also be used as project-specific internal reference for follow-up steps.[1] However, care must be taken when changing experimental conditions to ensure compatibility.

Regardless of the references to be used, they should always be assessed against each screening target to rule out binding of the reference to the target or even destabilization of the latter. To streamline the process, a selected subset of references can be pooled together and tested against the protein/nucleic acid of interest before launching screening efforts.

MATERIALS AND METHODS

Compounds and Libraries. All compounds investigated in this work were ordered from external vendors. The suppliers

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and catalog numbers are provided in the Supporting Information. The fragment library used to assess pool effects was provided by NMX Research and Solutions Inc. (Fast-Screen ^{19}F Fragment Library).

NMR Sample Preparation. Each compound was prepared as a 30 mM stock solution from the purchased powder or liquid in either dimethyl sulfoxide-d_6 or deuterium oxide (D_2O). This solution was then diluted to give the desired final compound concentrations. NMR samples were stored at 4 °C in a SampleJet sample handler, and data were acquired at 25 °C unless otherwise specified.

NMR Experiments. All experiments were run on a 600 MHz Bruker Avance III spectrometer equipped with a helium HFCN cryoprobe. 1D ^{1}H-decoupled ^{19}F experiments were acquired using the standard Bruker sequence zgfhigqn. Spectra were acquired with 32 scans and a relaxation delay of 10 s. 1D ^{1}H spectra were acquired using standard Bruker 1D ^{1}H sequence with excitation sculpting (zgesgp) and a relaxation delay of 10 s. Spectra were acquired with 16 scans.

To avoid potential interference with another internal reference, a deuterium lock on the magnet was used for ^{19}F referencing. To ensure sufficient robustness of this method, repeated measurements were performed on three samples containing 200 μM trifluoroethanol over three time points (0, 12, and 24 h). Very good consistency was observed across time points, with average variations in chemical shifts below 0.1 Hz (Table S1).

Data Interpretations. Data visualization and analysis were done in Bruker's TopSpin software (https://www.bruker.com/en/products-and-solutions/mr/nmr-software/topspin.html).

Proteins. All proteins used in this report were purchased from external vendors. The suppliers and catalog numbers are provided in the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00613.

Additional NMR figures; spectrometer stability of indirect referencing; information on compounds and proteins used in this report (PDF)

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Notes

The authors declare no competing financial interest.

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