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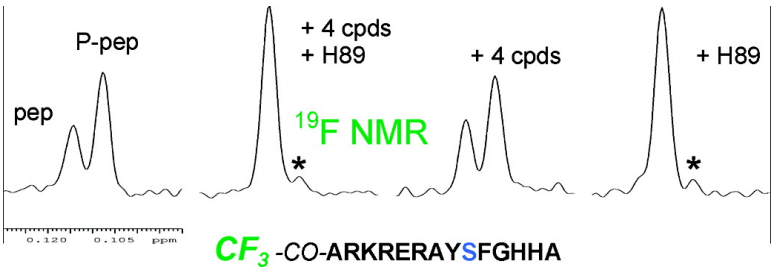

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A General NMR Method for Rapid, Efficient, and Reliable Biochemical Screening

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Abstract: High-throughput screening is usually the method of drug-lead discovery. It is now well accepted that, for a functional assay, quality is more important than quantity. The ligand-based or protein-based NMR screening methodologies for detecting compounds binding to the macromolecular target of interest are now well established. A novel and sensitive NMR method for rapid, efficient, and reliable biochemical screening is presented. The method named 3-FABS (three fluorine atoms for biochemical screening) requires the labeling of the substrate with a CF_3 moiety and utilizes ^{19}F NMR spectroscopy for the detection of the starting and enzymatically modified substrates. The method allows for high-quality screening of large compound or natural product extract collections and for measuring their IC_{50} values. Applications of this technique to the screening of inhibitors of the Ser/Thr kinase AKT1 and the protease trypsin are presented. In addition, an interesting application of 3-FABS to functional genomics is also presented.

Introduction

Pharmaceutical industries rely heavily on high-throughput screening (HTS) for the identification of potential drug candidates.[1,2] Screening based on homogeneous fluorescence methods[3,4] and the traditional SPA (scintillation proximity assay)[5] are nowadays the technologies of choice for a biochemical assay. The sensitivity and miniaturized format of these methodologies allow for the screening of large proprietary compound collections. However, a number of important issues such as the quality of the assays and the quality of the compound collection have emerged. The complexity of the assays utilized in HTS and interferences from the method of detection often result in a large number of false positives, i.e., hits that are then not confirmed in a subsequent secondary assay. In addition, HTS can fail at detecting potential inhibitors due to the large experimental errors and due to the impossibility of directly analyzing the concentration, purity, stability, and aggregation state of the screened compounds. It is now well accepted that, for screening, quality is more important than quantity.[1] Therefore, ways of making the biochemical assays for identification of new lead molecules more reliable and robust are in continuous development.

Over the past few years NMR has emerged as a powerful means for lead molecule identification. As these methods become increasingly important, several review articles on these subjects have appeared in the literature.[6−16] NMR is able to identify the true ligands and, therefore, coupled with HTS, can eliminate the false positives.[16,17] The power of NMR in this research area has begun to be recognized, and in some pharmaceutical companies,[17] only the hits confirmed by NMR are prioritized for further medicinal chemistry or combinatorial chemistry activities. NMR-based screening methodologies have been used mainly for identifying molecules that interact with the receptor. Although NMR has been applied extensively for characterizing the products of an enzymatic reaction or to gain insight into the kinetics of the reactions,[18−20] NMR-based biochemical screening that measures the inhibition or the activation of an enzyme for the identification of new lead

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molecules has found limited applications.[21] The low sensitivity of the proposed method limits the application only to enzymatic reactions with very high substrate concentration.

In this work, we present a novel and sensitive NMR method named 3-FABS (three fluorine atoms for biochemical screening) that allows for rapid and reliable biochemical screening performed at protein and substrate concentrations comparable to the ones utilized by standard HTS techniques. In a setting where the enzymology data are lacking or slow, one could envision a system being set up in the NMR lab from which chemists could obtain rapid values for the IC_{50} of their compounds using 3-FABS. In addition, these methods are extremely useful in an academic laboratory setting, where HTS instrumentation and expertise are simply not available.

Results and Discussion

Procedure. The technique requires the labeling of the substrate with a CF_3 moiety. This is easily achieved when the substrate is a peptide. (i) The peptide can be N-terminal capped with trifluoroacetic anhydride resulting in a N-trifluoroacetylated peptide, (ii) the CF_3 group can be inserted at the C-terminus via amide formation with a trifluoromethylamine, or (iii) a chemically protected amino acid with a CF_3 group is used in the peptide synthesis. For nonpeptidic substrates chemical synthesis is required for the introduction of the CF_3 moiety in the appropriate position of the substrate.

The enzymatic reaction is performed with the CF_3 labeled substrate and quenched after an established delay that depends on the enzyme, cofactors and substrate concentrations and the catalytic constant K_{cat}. For screening and IC_{50} measurements, this delay should be in the time region where the reaction is still linear. The enzymatic reaction can be quenched either by denaturating the protein, by chelating the cofactors, or by adding a strong known enzyme inhibitor.

Fluorine NMR spectroscopy is then used to monitor the starting and enzymatically modified substrate as shown in Figure 1 for two different substrates **1** and **2** of the AKT1 Ser/Thr protein kinase.[22,23]

CF_3-CO-ARKRERAY**S**FGHHA

(1)

ARKRERAF(*3-CF_3*)**S**FGHHA

(2)

Protein kinase AKT1 is an anti-apoptotic protein kinase that has an elevated activity in a number of human malignancies. The substrates represent simple modifications of the AKTide peptides.[24] This NMR method has some unique advantages: (i) Compared to other nuclei fluorine NMR spectroscopy is very sensitive, 0.83 times that of the proton. (ii) The CF_3 signal for a small to medium size substrate is a sharp singlet resonance (the experiments do not require proton decoupling if the CF_3 is not scalar coupled to protons) originating from three fluorine atoms, and therefore, the signal is very intense. This property

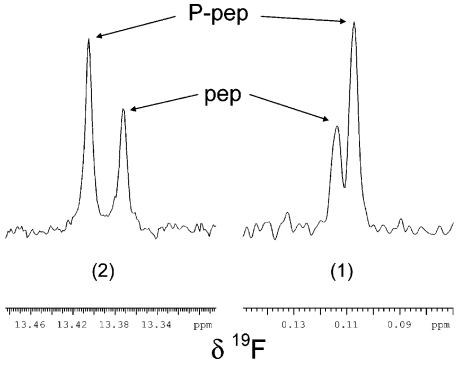

Figure 1. ^{19}F NMR spectra recorded for two different CF_3-labeled (AKTide like) substrates **1** (right) and **2** (left). The incubation time for the enzymatic reaction was 165 min with a protein, ATP, and CF_3-labeled substrate concentration of 25 nM, 131 μM, and 30 μM, respectively. The reaction was stopped by the addition of 10 μM of staurosporine, a very potent inhibitor (IC_{50} in the low nanomolar range). The samples (550 μL in volume) were in 50 mM Tris, pH 7.5, with 1 mM DTT and 5 mM $MgCl_2$. A total of 256 scans with 2.8 s repetition time were recorded for each spectrum (12 min measuring time). Chemical shifts are referenced to trifluoroacetic acid. Pep and P-pep are the substrate and the product, respectively. The difference in chemical shift between the starting and modified peptide is larger for the peptide on the left due to the closer proximity of the CF_3 group to the phosphorylation site.

is crucial for the screening because it permits the use of substrate concentrations that are in the range of its K_M thus allowing detection of medium and weak inhibitors. (iii) The ^{19}F isotropic chemical shift is extremely sensitive to small structural perturbations[25] resulting in different chemical shifts for the signals of the starting and enzymatically modified substrate even when the CF_3 is located far away from the site of modification as shown in the example of Figure 1 right. (iv) There are no spectral interferences from protonated solvents, buffers, and detergents typically present in the cocktail used for the enzymatic reactions. Overlap with the signals of the substrate in the presence of CF_3-containing molecules represents an extremely rare event due to the limited number of sharp singlet ^{19}F signals (molecules will typically have only one CF_3 group) and the large dispersion of ^{19}F chemical shift.

The speed of the enzymatic reaction for the two substrates in Figure 1 is very similar (0.9:1 left:right). Therefore, the substitution of the tyrosine of AKTide adjacent to the serine phosphorylation site with a phenylalanine bearing a CF_3 group in meta position of the ring does not alter significantly the binding affinity of the peptide to the enzyme. The peptides bind in an extended configuration (Elena Casale, personal communication) with the serine pointing toward the protein and its two adjacent residues pointing toward the solvent. Therefore, small modifications of the side chains of the two residues adjacent to the serine will not change significantly the binding constant of the substrate.

Screening, Deconvolution, and IC_{50} Measurements. Before performing the screening, it is important to measure the binding constant of the substrate or substrates.[26] This is necessary for properly setting up the substrate concentrations to be used in the screening process and for deriving the binding constant K_I of the identified inhibitor from its IC_{50} value according to the following equation:[27]

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$$K_I = \frac{IC_{50}}{1 + \frac{[S]}{K_D}} \quad (1)$$

Here [S] and K_D are the concentration and the dissociation binding constant of the substrate, respectively.

Enzymatic reactions are performed at different substrate concentrations, and the CF_3 signal of the modified substrate is monitored as shown in Figure 2 for ATP binding to AKT1. A plot of the signal intensity of the product as a function of the substrate concentration allows the determination of the binding constant of the substrate (see Figure 2). An alternative is to plot the speed of the enzymatic reaction (^{19}F signal intensity of the product divided by the incubation time) as a function of the substrate concentration. For the latter, incubation times of the enzymatic reaction can differ for the different samples.

Compounds are typically screened in small or large mixtures, as shown in the example of Figure 3, top, at a concentration that will depend on the desired detection threshold. For example if only strong inhibitors with $IC_{50} < 5$ μM will be considered, then it will be sufficient to use molecules at a 5 μM concentration. In practice, the reliability of 3-FABS allows the identification of weaker inhibitors with an IC_{50} in the range 10−20 μM even when the concentration of the screened molecules is only 5 μM. This low concentration allows the preparation of mixtures with a large number of components without severe problems of aggregation and solubility. Deconvolution of the active mixtures is then carried out for the identification of the inhibitor (Figure 3, top). For the determination of the IC_{50} of the hits, experiments at different inhibitor concentrations are then performed and the integral of the ^{19}F signal of the product or of the starting substrate (when the concentration of the substrate is the same in all samples) is measured. A plot of these values as a function of the inhibitor concentration allows the determination of IC_{50} as shown in Figure 3, bottom, for the compound H89. The IC_{50} values that can be measured with precision range from few nanomolar (limit determined by the protein concentration) to millimolar (limit determined by the compound concentration). In the absence of allosteric effects a meaningful IC_{50} value is derived with a single point measurement. This is possible because the values of both plateaus are known. These are (i) the concentration of the unmodified substrate in the absence of the inhibitor and the total substrate concentration when the ^{19}F signal of the unmodified substrate is monitored and (ii) the concentration of the modified substrate in the absence of the inhibitor and the 0 value when the ^{19}F signal of the modified substrate is monitored. However, it should be stressed that the single point measurement provides only an approximate IC_{50} value. A CF_3 labeled ATP analogue can also be used with 3-FABS. In this case, the ^{19}F signals of ATP and ADP are monitored. However, the hydrolysis of ATP that is not due to the kinase reaction might affect the accuracy of the IC_{50} measurement.

The screening can be performed at different ATP or peptide concentrations. These concentrations can be selected according to eq 1 for facilitating the identification of inhibitors that bind in the ATP binding pocket or inhibitors that compete with the peptide for the substrate binding site.

The 3-FABS probably represents the simplest possible biochemical assay because it is homogeneous and detects directly both starting and modified substrate. It does not require (i) the presence of secondary reactions performed with enzymes or specific antibodies or (ii) separation and/or washing steps necessary for the readout with other methods. The simplicity of 3-FABS results in reliable lead molecule identification and quantification of their inhibitory activity. Even compounds displaying only a weak inhibitory activity can be safely selected. These molecules would often be discarded in the analysis of the HTS hits because the values of inhibition are contained in the error bars of the measurements. The possibility of unequivocally identifying these weak inhibitors known also as *latent hits*[28] is very important when only a limited chemical library of compounds is available for screening. Small chemical changes of the weak inhibitors or the selection of similar molecules bearing the same scaffold might result in the identification of potent inhibitors.

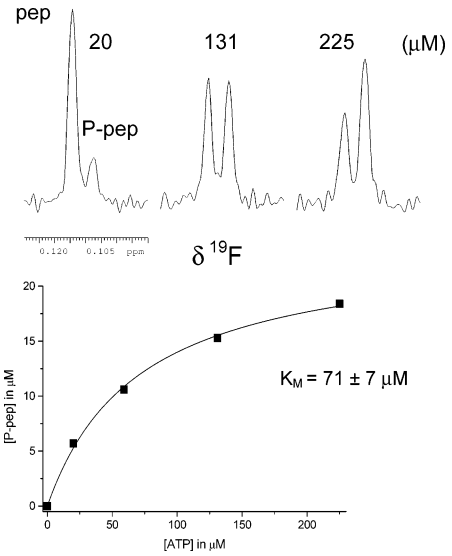

Figure 2. K_M measurement of ATP. Top: ^{19}F NMR spectra for **1** recorded with three different concentrations (indicated in μM) for ATP. The activated protein and the peptide concentrations were 50 nM and 30 μM, respectively. The reaction was run at 22 °C and quenched after 60 min with the addition of 10 μM staurosporine. Two replicate measurements were performed. Bottom: Phosphorylated peptide concentration (measured with integration of the ^{19}F NMR signal) as a function of the ATP concentration and the best fit of the experimental data with the derived K_M for ATP of 71 ± 7 μM (compared to the value of 66 μM determined with enzymology). Note that the K_M in a Michaelis−Menten mechanism corresponds to the dissociation binding constant K_D of the enzyme−substrate complex.

The experiments can be compared in their simplicity to binding experiments performed with fluorescence spectroscopy or ITC measurements. Therefore, the obtained results are highly reproducible. In addition, an advantage of NMR applied to biochemical assays when compared to other techniques used in HTS is the possibility to directly characterize with NMR the screened compound. Often the real sample concentration differs from the nominal concentration. A large difference in compound concentration results in a significant error of the derived IC_{50} value. The causes for the concentration differences can be ascribed to weighing errors, sample impurity, poor solubility of the compound, and chemical instability in an aqueous environment. These chemical properties can be easily measured with NMR by acquiring, in addition to the fluorine spectrum, also a proton spectrum. The real concentration of the inhibitor

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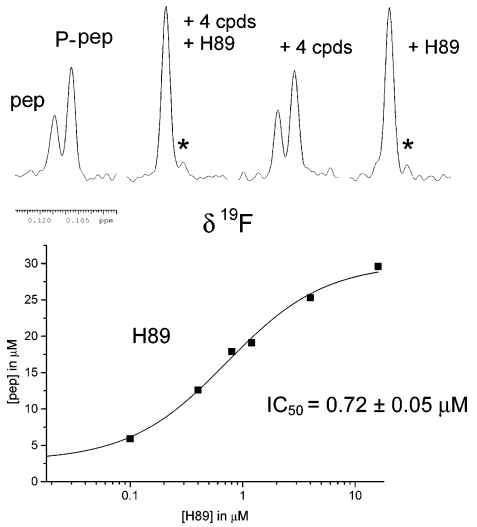

Figure 3. Screening and deconvolution (top) and IC_{50} measurement (bottom). Top: ^{19}F NMR spectra for **1** recorded from left to right in the absence of compound, in the presence of a five molecule mixture (2-amino-6-methylquinazolin-4-ol, ethyl 2-quinoxalinecarboxylate, 5-methylbenzimidazole, methyl isoquinoline-3-carboxylate, and *N*-(2-{[(2*E*)-3-(4-bromophenyl)prop-2-enyl]amino}ethyl)isoquinoline-5-sulfonamide known as H89[31]), in the presence of the same mixture without H89, and in the presence of only H89. The activated protein, the peptide, ATP, and compounds concentrations were 25 nM, 30 μM, 131 μM ($\sim 2K_M$), and 10 μM, respectively. The asterisks indicate the tiny amount of phosphorylated peptide. Bottom: Unphosphorylated peptide concentration (measured with integration of the ^{19}F NMR signal) as a function of the inhibitor concentration and the best fit of the experimental data with the derived IC_{50} for H89 of 0.72 $\pm$ 0.05 μM (compared to the value of 0.5 μM determined with enzymology). The same IC_{50} value was obtained using the integral of the ^{19}F signal of the phosphorylated peptide. All the reactions (top and bottom) were run at 22 °C and quenched after 120 min with the addition of 10 μM staurosporine. Two replicate measurements were carried out. In the absence of allosteric effects, a meaningful IC_{50} value is derived with a single point measurement as explained in the text.

determined with ^1H NMR allows thus an accurate measurement of its IC_{50} value.

The method is not limited to the identification of inhibitors only but can also be used for the detection of agonists. In the specific case of kinases the ^{19}F signal of phosphorylated peptide in the presence of an agonist is larger when compared to the same signal of the reference sample (i.e. sample for which the reaction was performed in the absence of compounds to be screened).

Protein Requirement, Detection Limits, and Throughput. The concentration of the protein used with this method is solely determined by the speed of the enzymatic reaction. Protein concentrations as low as a few nanomolar can be used, therefore comparing favorably with the concentration used in HTS. However, the volume necessary for each NMR sample using a 5 mm probe is about 500−550 μL. A 2- to 3-fold volume reduction is achieved with the use of a flow-injection probe or a 3 mm probe. The high sensitivity of the CF_3 signal allows for rapid acquisition of the spectra. In our screening efforts spectra are recorded with only 64 scans (see Supporting Information) resulting in a 3 min acquisition time. The same spectra can be recorded more rapidly with the use of cryogenic technology applied to ^{19}F detection. A 4-fold improvement in the sensitivity achieved with this technology translates into a 16-fold reduction in the measuring time. Therefore, the spectra that require with our current settings an acquisition time of 3 min can be recorded in just 12 s. It should be pointed out that problems of radiation damping encountered in proton-detected experiments recorded with cryoprobes are absent in the fluorine-detected experiments because of the low concentration of the CF_3-labeled substrate. The rapid spectral acquisition and the utilization of compound mixtures allow for the high throughput necessary to screen large proprietary compound collections. A further improvement in the throughput can be expected with the utilization of a flow-injection ^{19}F cryoprobe. Finally, the sensitivity gain achieved with the cryoprobe technology optimized for ^{19}F detection will allow in some fortunate cases the utilization of 1-FABS where the substrate is labeled with a CF moiety.

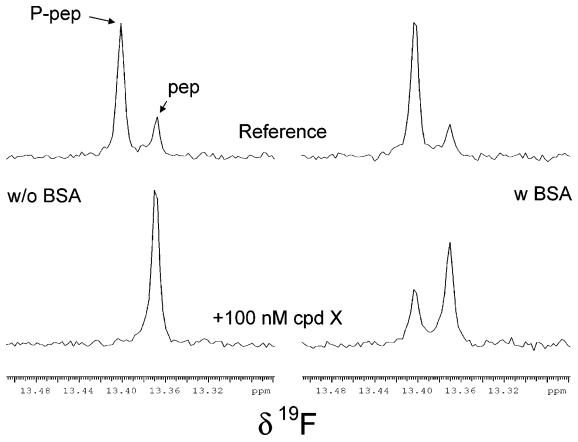

Figure 4. Influence of BSA on the IC_{50} measurement: ^{19}F spectra of **2** in the absence (left) and presence (right) of BSA. The reactions were performed in the absence (top) and presence (bottom) of 100 nM inhibitor. The incubation times were selected so that the amount of phosphorylated peptide was the same in the presence and absence of BSA for the top spectra. The incubation time was 200 min (left) and 80 min (right). The activated protein AKT1, the peptide, ATP, and BSA concentrations were 25 nM, 30 μM, 131 μM, and 3 μM, respectively. A concentration of 100 nM of the compound was sufficient for totally inhibiting the reaction in the absence of BSA. However a drastic reduction of the enzyme inhibition is observed in the presence of BSA due to the high affinity of the molecule for BSA.

Data analysis is fast and straightforward. For this purpose it is sufficient to measure the intensity of the two ^{19}F signals and take their signal intensity ratio (this is valid only if the same substrate concentration is used in all samples). The deconvolution efforts are then directed at those mixtures where the intensity ratio value differs from the value recorded for the reference sample (i.e. sample without the presence of chemical mixtures). Complete automatic IC_{50} determination is also possible with this approach.

Screening in the Absence and Presence of Bovine Serum Albumin. At the low protein concentration used for the NMR screening, problems are encountered with the sticking of the protein to the tube wall.[29] In our experiments reactions are performed in the Eppendorf vials, and after the quench of the reaction the solution is transferred to the NMR tube. To prevent reagent coating BSA (bovine serum albumin) is often added to the solution. The enzymatic reaction in the presence of BSA becomes faster because all the enzyme is available in solution. However, the presence of BSA used in large excess compared to the enzyme (typically > 100-fold excess) can significantly alter the IC_{50} measurement by sequestering the compound from the solution, as shown in the example of Figure 4. The IC_{50} of the compound in the presence of 3 μM BSA was about 75 times weaker when compared to its IC_{50} in the absence of BSA.

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Different compounds bind with different affinities to BSA, and therefore, a meaningful SAR will not be possible. Compounds binding strongly to the enzyme might actually display a weaker inhibitory activity due to their high affinity to BSA. In our experience we found examples where the ranking of the compounds of a defined chemical class according to their IC_{50} values was different in the absence and presence of BSA (data not shown). Therefore, 3-FABS performed in the absence and presence of BSA, or better HSA (human serum albumin), for the compounds in the hit to lead optimization phase, can provide early on in the project important structural information for designing analogues with retained inhibitory activity to the target enzyme and reduced affinity to albumin.

Do It Yourself IC_{50} Measurement. The method allows for the construction of a meaningful and robust structure and activity relationship table around a chemical class of compounds in development. As discussed previously, the generated IC_{50} values with 3-FABS are less subject to artifacts originating from interferences of the detection system. In addition, NMR allows also the determination of the inhibitor concentration and its purity and solubility, thus resulting in a more accurate measurement of the IC_{50} value. The "easy to use" property of the method permits its utilization also to people not expert in the NMR field. It is conceivable that one day the chemist who uses NMR to characterize newly synthesized molecules will then use the same technology for measuring the inhibitory activity of each molecule against a target enzyme. A 300−400 MHz spectrometer will be sufficient for the analysis when the difference in ^{19}F chemical shift between starting and enzymatically modified substrate is large, as for the substrate **2**. A higher field spectrometer of 500−700 MHz is required when the difference in chemical shift is small, as for the substrate **1**. A probe with the inner coil tuned to ^{19}F and the outer coil tuned to ^{1}H is recommended. This configuration allows for maximum sensitivity of the ^{19}F nucleus detection. The ^{1}H coil is used for proton decoupling when needed and for recording proton spectra of the NMR hits or the newly synthesized molecule to confirm their structure and determine their concentration and sample purity.

When the substrate molecule is stable, NMR spectra can be recorded even several days after the quench of the enzymatic reaction. Therefore, the chemist can queue his/her samples in an open access NMR instrument and analyze the data (or receive the already analyzed data as discussed above) the day after or after the weekend.

Other Applications and Functional Genomics. (i) The method can be applied to many different types and subtypes of enzymes (e.g. proteases, phosphatases, ligases, etc). This can be appreciated with the example of Figure 5. The screening was performed with trypsin, a protease that cleaves peptide bonds C-terminally of lysine and arginine[30] and using, as a substrate, the peptide **2**. In the presence of trypsin two ^{19}F signals are visible in the spectrum (Figure 5b) at 13.38 and 13.33 ppm originating from the starting peptide and the cleaved peptide, respectively. Screening was performed in an end point assay format where the reaction was quenched after a defined delay using a known trypsin inhibitor. The peptide was not cleaved in the presence of a five compound mixture (Figure 5c).

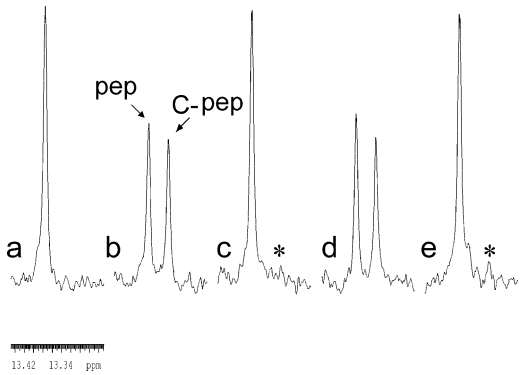

Figure 5. Screening with proteases, where the substrate is the peptide **2** and the protein is trypsin from bovine pancreas: ^{19}F NMR spectra for the peptide in the absence (a) and in the presence (b−e) of trypsin. The reaction was run at 22 °C and quenched after 30 min with the addition of 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The reaction was performed in the absence of compound (b), in the presence of a five compound mixture (2-amino-6 methylquinazolin-4 ol, ethyl 2-quinoxalinecarboxylate, 5-methylbenzimidazole, methyl isoquinoline-3-carboxylate, and leupeptin) (c), in the presence of the same mixture without leupeptin (d), and in the presence of only leupeptin (e). The protein, peptide, and compound concentrations were 15 nM, 30 μM, and 20 μM, respectively. A total of 128 scans with 2.8 s repetition time were recorded for each spectrum (6 min measuring time). Pep and C-pep are the substrate and the product, respectively. The asterisks in (c) and (e) indicate the chemical shift of the missing cleaved peptide signal.

Deconvolution of the active mixture was then performed for the identification of the inhibitor (Figure 5d,e).

(ii) The method can also be used for the selection of the most efficient CF_3-labeled substrate as shown in the example of Figure 1. (iii) Natural product extracts can be easily screened with this method. The components of the active extracts will then be separated via HPLC and tested as single compounds with 3-FABS for the identification of the compound responsible for the inhibitory activity.

(iv) Another potential application of the method is in the determination of protein function. In today's era of high-throughput genome sequencing, thousands of new proteins have been identified. However, the potential of these proteins as drug targets cannot be determined without knowledge of their function. It is possible to infer the function of a protein on the basis of what types of substrates are modified. For this purpose, a functional genomic library of CF_3-labeled substrates of enzymes of known function is generated. These substrates are first tested on their known respective enzymes to determine the ^{19}F chemical shifts of the starting and modified substrate. Some compounds, such as the example of Figure 6, can act as substrates for different classes of enzymes. This library of substrates is then screened as single compounds or in small mixtures against the protein with unknown function. The reduction in signal intensity of a ^{19}F signal and the appearance of a new resonance at a defined chemical shift allows the recognition of the protein function. This is shown in the test case of Figure 6. The lower spectrum was recorded for the protein PAK-4. A simple comparison of this spectrum with the other two allows the classification of this protein to the kinase family. A higher concentration for the protein with unknown function and a longer incubation time are clearly required for these experiments because the substrate is not optimal for the protein with unknown function. In the example of Figure 6 the speed of the enzymatic reaction of PAK-4 is only 1/22 of the

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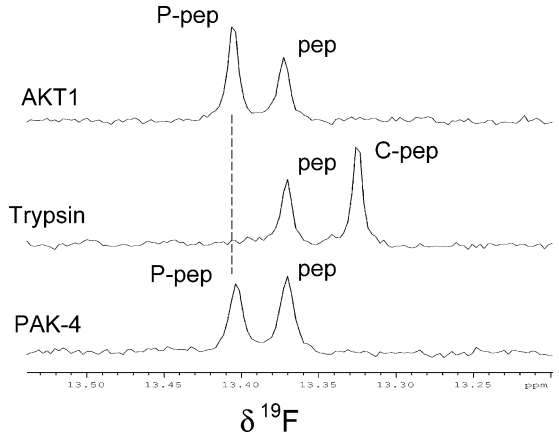

Figure 6. Application of 3-FABS to functional genomics with ^{19}F spectra of **2** in the presence of three different proteins, 25 nM AKT1 (top), 15 nM trypsin (middle), and 150 nM PAK4 (bottom). The peptide is a good substrate for AKT1 and for trypsin. The chemical shift of the cleaved peptide is quite different when compared to the chemical shift of the phosphorylated peptide. The reaction performed in the presence of the p21-activated protein PAK4 results in the appearance of a signal at the chemical shift of the phosphorylated peptide.

speed of AKT-1. Nevertheless, it is sufficient to detect a small signal of the modified substrate for deriving the protein function.

This method is clearly limited to activated enzymes. For a more general application it is sufficient to complement, the CF_3-labeled substrates library with a collection of CF- or CF_3-labeled known ligands. FAXS[32] and 3-FABS are then used for the screening against the proteins with unknown function. The technique has worked well for the example of Figure 6, but currently we do not know if it represents a general method. More work is needed to fully evaluate the potential of 3-FABS applied to functional genomics.

Conclusions

Over the past few years, NMR-based screening has been used for the identification of protein ligands. The 3-FABS presented here extends the capabilities of NMR allowing for rapid, powerful, and reliable biochemical assay for the identification of inhibitors. The method coupled with some of the now well-established ligand-based and/or protein-based NMR screening experiments represents a powerful tool in the hand of researchers for lead identification and lead optimization. The sensitivity and reliability of 3-FABS allows for high-quality screening of large compound or natural product extract collections. Its simplicity in setup, run, and readout aspects permits the utilization also to people not expert in the NMR or biochemical screening fields. Spectra can be recorded in just a few seconds using the cryogenic technology applied to probes with ^{19}F detection, thus allowing rapid screening and IC_{50} measurements. Thus, the technique should find numerous uses in the pharmaceutical industry and should further extend the impact of NMR on drug discovery.

In addition, these methods are extremely useful in academia or small companies, where HTS instrumentation and expertise are simply not available. Another potential application of the method is in functional genomics for inferring the function of a newly sequenced protein.

Material and Methods

WtAKT1 recombinant protein has been produced by infection of Sf21 insect cells with baculovirus coding for the full length protein fused to GST at the N-terminus. The cells have been treated with okadaic acid for 4 h prior to harvesting. This treatment, by inhibiting the cellular phosphatases, increases the total phosphorylation level of the protein leading to the phosphorylation of the two sites critical for AKT activity, threonine 308 in the activation loop and serine 473 in the C terminal hydrophobic motif. Lysis, purification, and removal of the GST tag have been done by following standard procedures. Trypsin, purchased from Roche Molecular Biochemical (Cat. No. 1418025), was dissolved in 1% acetic acid solution at a final stock solution concentration of 8.33 μM.

The serine/threonine p21-activated kinase PAK4 was expressed as a GST fusion protein in *Escherichia coli* and purified to homogeneity after removal of the GST tag.

All the compounds were prepared in DMSO stock solutions (20−40 mM). The substrates **1** and **2** and Leupeptin were prepared in aqueous solutions at a concentration of 10 and 2.1 mM, respectively. The reactions were run in 50 mM Tris pH 7.5, with 1 mM DTT and 5 mM $MgCl_2$ for AKT1 and 50 mM Tris pH 7.5 for trypsin. D_2O was added to the solutions (8% final concentration) for the lock signal. The enzymatic reactions were performed at room temperature in Eppendorf vials and then quenched after a defined delay with the addition of staurosporine for AKT1 and PMSF for trypsin. The solutions were then transferred to 5 mm NMR tubes.

All NMR spectra were recordeded at 20 °C with a 600 NMR spectrometer operating at a ^{19}F Larmor frequency of 564 MHz. A 5 mm probe tunable to either ^{19}F or ^{1}H frequency was used. The instrument was equipped with a sample management system (SMS) autosampler for automatic data collection. The data were acquired without proton decoupling with an acquisition time of 0.8 s and a relaxation delay of 2.8 s. Chemical shifts are referenced to trifluoroacetic acid.

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Supporting Information Available: ^{19}F 564 MHz NMR spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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