Ligand Screening

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Phosphorus NMR Spectroscopy as a Versatile Tool for Compound Library Screening**

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Dedicated to Professor Wolfgang A. Herrmann on the occasion of his 60th birthday

NMR spectroscopy is a well-established technique for the screening of compound libraries.[1] One of the biggest advantages of NMR spectroscopy in relation to other methods is that it directly detects even weak interactions between ligand and target molecules, which makes it ideally suited for fragment-based ligand design. In addition, the number of false-positive hits, often obtained in bioassays, is minimized.[2,3] Among the variety of NMR screening approaches, methodologies based on exclusively tracing ligand signals are the most powerful tools to identify binders in compound libraries.[4] Standard and group-selective saturation-transfer difference (STD) spectroscopy or fluorine NMR screening are prominent examples.[5–8] Together with recently developed high-throughput techniques, for example, target-immobilized NMR screening (TINS),[9] ligand-based NMR screening is a potent technology in the field of drug science.[1,10–12]

To further enhance the applicability of NMR spectroscopy in biological and pharmaceutical research, we introduce phosphorus (^{31}P) as a new nucleus for compound library screening of protein inhibitors. As ^{31}P is a spin-$^1/_2$ nucleus with a gyromagnetic ratio of 17.24 MHz T^{-1} in natural abundance of 100%, it is widely used in magnetic resonance techniques, especially for in vivo NMR applications. In contrast, no ^{31}P-NMR based screening approach has appeared in the literature. However, phosphorus reveals several physical and chemical properties making it well suited for the utilization in library screening. For example, the problem of overlapping resonances normally arising in proton-detected spectra is reduced because each compound is represented by only one ^{31}P-signal in most cases. Depending on the oxidation state of phosphorus and the type of directly bound atoms, common organic phosphorus compounds show a wide chemical-shift dispersion of about 100 ppm, thus allowing the screening of huge compound libraries.

As ^{31}P nuclei exhibit large chemical shift anisotropy (CSA), even weak binding events can easily be traced owing to a strong T2 relaxation-rate-dependent line broadening of the affected ligand signals. Moreover, phosphorus is often an intrinsic element of compounds mimicking the tetrahedral intermediate of a peptide bond hydrolysis (often called transition-state analogues; see Figure 1), for example, in protease inhibitors.[13–17] Thus, interactions with proteases are detected without the auxiliary ligand labeling[7] that is often required for ^{19}F based screening methods.

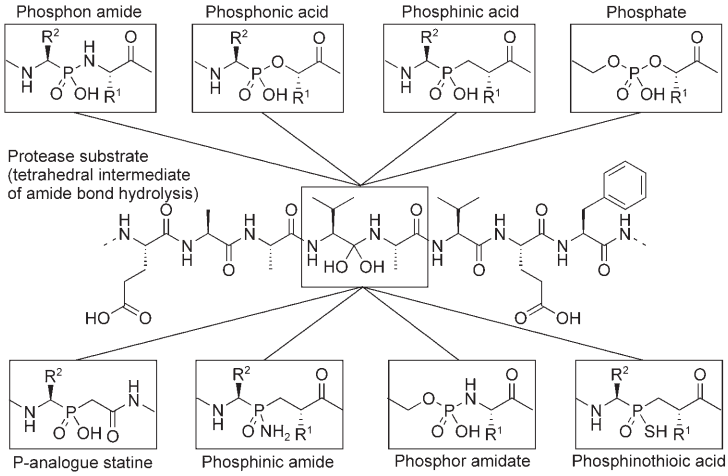

Figure 1. Possible phosphorus-containing isosteres for the hydrolyzable peptide bond in rational protease inhibitor design.

Molecules containing phosphorus play not only an important role in proteases, but also in the wide range of biochemical pathways, for example, in phosphoryl transfer reactions. After identification of a small phosphorylated substrate, this ligand (or a more stable derivative) may serve as reporter molecule in the screening for new inhibitors. Scheme 1A gives some examples for this strategy.[18–21] In addition, various phosphorus-containing drugs were approved by the FDA in recent years (Scheme 1B). To break through the obstacle of poor oral availability, several of these agents were developed as prodrugs (for example, Monopril).

The fundamental soundness of the presented method was established by a series of NMR experiments. We chose the thermolysin–phosphoramidon system, which has been inves-

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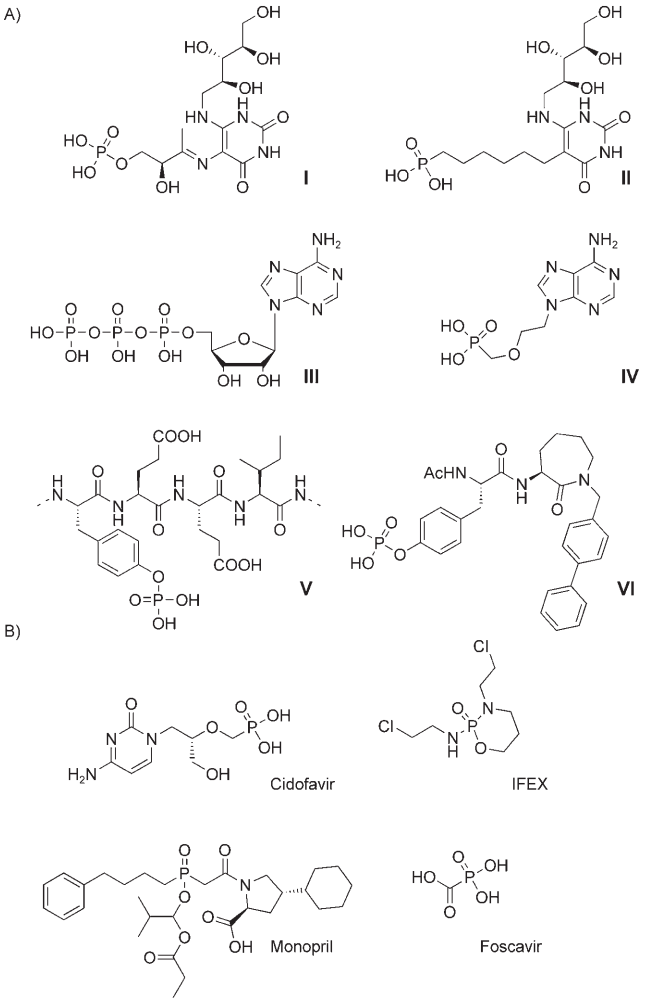
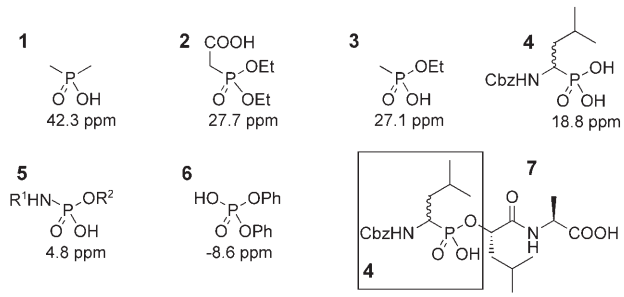


Scheme 1. A) Natural phosphorylated substrates (PS) and their phosphorus-containing analogues (PCA) as possible reporter molecules for compound library screening: PS of lumazin synthase (**I**) and PCA (**II**) as inhibitor of lumazin synthase. ATP (**III**) and its nucleotide analogues, such as AMP-PNP (not shown) or Hepsera (**IV**), which is a bioavailable drug used for treatment of hepatitis B by inhibition of reverse transcriptase. PS of Src protein (**V**) and PCA (**VI**) as inhibitor of Src SH2 kinase domain. B) Examples of approved ^{31}P-containing drugs: Cidofavir is an injectable, antiviral agent for the treatment of *Cytomegalovirus retinitis*. IFEX is a nitrogen mustard alkylating drug used in the treatment of cancer. Monopril is an angiotensin converting enzyme inhibitor for medicating hypertension and chronic heart failure. Foscavir is an antiviral agent to treat herpes virus deseases.



Scheme 2. Structures and chemical shifts of compounds used (**1**–**6**) are shown along with CbzL^{P}(O)LA (**7**) containing **4** as a fragment. R^{1}: L-leucyl-L-tryptophan, R^{2}: L-rhamnopyranosyloxy(hydroxyphosphinyl).

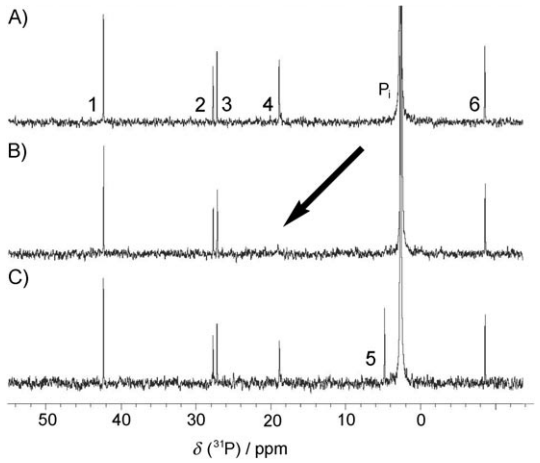


Figure 2. ^{31}P NMR spectra of a small ligand library (each 0.5 mM) in phosphate buffer (pH 7.5) containing 3 M KBr to keep thermolysin in solution A) before and B) after addition of thermolysin (0.25 mM). The signal of **4** disappears in the presence of protein (see arrow). C) Recovery of the vanished signal upon addition of the tight binder phosphoramidon (0.5 mM). Effects on the signal line width that allow identification of binders can be seen with less than 1% of the protein concentration used herein (see Figure 3). P$_i$ = buffer signal.

tigated in detail.[22] Thermolysin (~35 Da) is a thermostable, calcium-binding zinc endopeptidase isolated from *Bacillus thermoproteolyticus*. Phosphoramidon, a natural product first isolated from *Streptomyces tanashiensis*,[23] contains a phosphoramidate unit as isostere of the hydrolyzable peptide bond. It binds to thermolysin with a K_i value of 28 nM.[24] To complete our small but representative molecule library, five substances displaying some of the isosteres shown in Figure 1 were selected (Scheme 2).

A proton-decoupled ^{31}P-1D-NMR spectrum of an equimolar mixture of **1**–**4** and **6** (each 0.5 mM) was recorded, and is shown in Figure 2A. We utilized hydrogen phosphate as buffering agent and internal pH control. As both the resonances exhibit large chemical-shift dispersion, and each compound is represented by only one signal, the spectrum of the library is simple, and every peak was easily assigned. Upon addition of thermolysin to a final concentration of 0.25 mM, the signal of compound **4** almost completely vanishes, whereas all other resonances remain unaffected (Figure 2B). Thus, a new ligand for thermolysin was identified. Owing to the ligand–receptor interaction, the small compound adopts the fast T2 relaxation time of the protein. This causes a strong line broadening, finally leading to the disappearance of the ligand resonance. The identified binder turned out to be a fragment of the molecule CbzL^{P}(O)LA (**7**, Cbz = benzyloxycarbonyl) which was introduced by Bartlett and Marlowe as a thermolysin inhibitor (Scheme 2).[25] Thus, the described screening technology is able to detect small binders ideally suited for fragment-based drug design (FBDD).[26,27]

Owing to the large CSA of phosphorus nuclei, which leads to significantly broadened signals pertaining to the bound ligand, the new approach may be vulnerable to false-positive hits when unspecific interactions between a compound and the target are present. To overcome this drawback, and to rule out ligand binding to an undiscovered second site of thermolysin, the signal of **4** must be restorable by adding a known, high-affinity substance to the library. Herein we used the strongly binding phosphoramidon (**5**) to compete with **4**. As can be seen in Figure 2C, the addition of **5** to a final concentration of 0.5 mM results in an almost complete recovery of **4**, whereas the signal of free phosphoramidon appears at $\delta = 4.8$ ppm. This result clearly shows that **4** binds exclusively at the active site of thermolysin and that **5** competes with **4** for a specific interaction with the protein. It should be noted that this approach can also be used for reporter-based screening. This means that a phosphorus-containing molecule (see Scheme 1) that weakly binds to a protein is monitored by ^{31}P NMR while a substance library of any constitution is screened with regard to competition. As soon as the resonance of the reporter molecule is recovered, a ligand with equal or higher affinity to the target is found.

To evaluate the sensitivity of phosphorus-based NMR screening, the dependence of the peak height of the binding compound on various protein concentrations was examined (Figure 3). Although the ligand (0.5 mM starting concentration) is present in large excess over the protein, the observed resonance of **4** almost completely vanishes upon addition of small amounts of thermolysin (Figure 3). To be more precise, the presence of only 6 μM protein leads to a reduction of the ligand signal to half its original height. As a result of the large CSA of phosphorus nuclei, a strong line broadening of the compound signal is induced by complex formation. Thus, solely low micromolar or even submicromolar protein concentrations are required for the facile identification of an unknown binder with ^{31}P NMR screening. Considering the titration plot (Figure 3) and the graphical analysis (see the Supporting Information, Figure S1), it becomes clear that ligand-based phosphorus NMR screening is a very sensitive method for probing intermolecular interactions between proteins and small- or medium-sized ligand molecules. Therefore, the NMR screening process is enhanced by both shortening the time and lowering the costs of protein production.

Although ^{31}P NMR resonances show a wide chemical shift range (more than 100 ppm), signal overlap may arise when working with libraries consisting of similar substances. One way to reduce or even overcome this problem is by performing heteronuclear two-dimensional measurements. As an example we recorded 2D-^{1}H,^{31}P-COLOC experiments (COLOC = correlation via long-range coupling) of our molecule library,[28] both with (in red) and without thermolysin (Figure 4). Considering only the ^{31}P dimension, the signals of

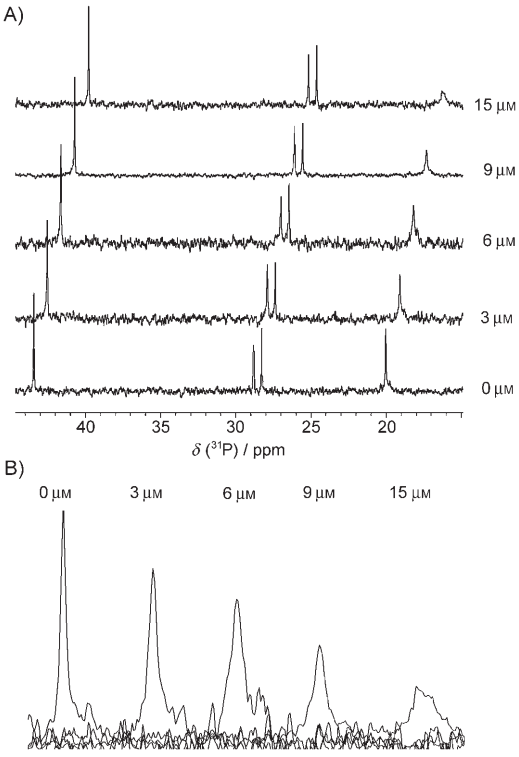


Figure 3. A) Section of a shifted overlay of several ^{31}P-1D-NMR-spectra. Compound library (**1**–**4**, 0.5 mM) without protein, and after addition of 3, 6, 9, and 15 μM thermolysin. B) An increase of protein concentration results in a strong line broadening of the affected resonance of **4**.

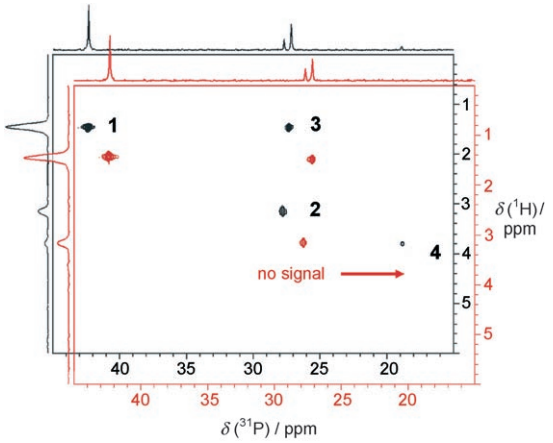


Figure 4. Section of an overlay of two 2D-^{1}H,^{31}P-COLOC spectra. Compound library (**1**–**4**, 0.5 mM) without (black) and with (red) 0.25 mM thermolysin. Addition of the protein leads to the disappearance of the cross-peak of **4**. The two phosphorus signals which are close to each other at ca. 27 ppm are well separated in the proton dimension.

2 and **3** are close to each other. Assigning those resonances may be difficult in cases when induced shift changes occur. However, the signals are clearly separated in the 2D spectrum. As it is the case in the one-dimensional spectrum, the disappearance of the peak for **4** in the red spectrum indicates binding. In addition, it is evident that signals with similar chemical shifts in both dimensions can also be discriminated by their cross-peak intensity, which is almost linearly related to the number of ^{31}P coupled protons via two-bond *J*-coupling (see Figure 4).

In conclusion, we demonstrated that phosphorus NMR spectroscopy enhances and even extends ligand-based screening of compound libraries. To show the broad applicability of ^{31}P NMR screening, we demonstrated not only a proof of principle of our concept but also valuable extensions of the method, such as recovery experiments and heteronuclear 2D-NMR measurements. As many substances that mimic the tetrahedral intermediate of peptide bond hydrolysis contain phosphorus (see Figure 1), ^{31}P NMR may be especially applicable to screen large mixtures of protease inhibitors. Furthermore, stable analogues of naturally phosphorylated substrates constitute powerful starting points for the design of ^{31}P-containing compound libraries (Scheme 1 A). However, mixtures that comprise non-phosphorus-containing substances can also be screened by utilizing a ^{31}P-containing reporter ligand. In contrast to other screening techniques, for example, bioassay-based methods that are exclusively focused on finding strong binders, phosphorus NMR screening also allows the identification of ligands with medium or even weak affinity to a target molecule. To diminish the number of false-positive hits, recovery experiments, and utilization of low magnetic fields in which unspecific binding causes only weak T2 relaxation-dependent line broadening, are suggested. Therefore, the approach presented herein is notably suited for application in the field of fragment-based drug design.

Experimental Section

All NMR experiments were carried out on a Bruker Avance 250 MHz spectrometer equipped with a 5-mm QNP probehead. The sample temperature in all measurements was 300 K, and 10% D_2O was used as the lock signal. 1D-^{31}P spectra were recorded with a standard Bruker pulse program, using a 30° excitation pulse, a 1.5 s recycle delay and a WALTZ-16 power-gated composite-pulse proton decoupling. For heteronuclear 2D experiments, the COLOC pulse program described in the literature was utilized.[28] The delay for evolution of long-range couplings was 25 ms. In each case, 4096 data points in the direct dimension were traced, and the spectral width was 70 ppm. For more details, see the Supporting Information.

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