influences, we believe that the crystal packing, contrary to that expected based on centrosymmetric dimer motifs, is most likely manifest in compounds, whose structures are 1) characterized by steric hindrance about the carboxy group and 2) endowed with certain interacting groups that are nonlinearly built into the molecules (at the *meta* position). We are continuing to investigate the self-assembly of aromatic carboxylic acids of varying structures and a variety of interactions.

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[23] Crystal data for **4**·H_2O: Slow evaporation of **4** in methanol–dichloromethane (2:1) mixture yielded suitable crystals for diffraction studies. The intensity data were collected on a Siemens P4 single-crystal diffractometer equipped with a molybdenum sealed tube ($\lambda =$ 0.71073 Å) and highly oriented graphite monochromator. The lattice parameters and standard deviations were obtained by a least-squares fit to 40 reflections ($9.58° < 2\theta < 25.55°$). The data were collected in 2θ-θ scan mode with a variable scan speed ranging from 2.0° to a maximum of 60.0° min^{-1}. The structure was solved by direct methods using SHELX-97 (G. M.Sheldrick, Program for the solution and refinement of crystal structures, University of Göttingen, Göttingen (Germany), **1997**) package and also refined using the same program. All the non-hydrogen atoms were refined anisotropically. The hydrogen atoms, except those of the water molecule, were stereochemically fixed on their ideal positions with fixed isotropic U values and their C–H distances were refined freely. The hydrogen atoms of the water molecule were identified from the difference Fourier transform and the O–H distances were varied freely during the refinement. A weighting scheme of the form $w = 1/[\sigma^2(F_o^2) + (aP)^2 + bP]$ was used. The difference Fourier map, after the refinement, was featureless. Crystal dimensions: $0.21 \times 0.18 \times 0.14$ mm; $T = 293(2)$ K; trigonal, $R\bar{3}$ (hexagonal axes); $a = b = 25.543(2)$ $c = 15.384(2)$ Å; $V =$ 8692.5(2) $Å^3$; $Z = 18$; $\rho_{calcd} = 1.184$ g cm^{-3}; $2\theta_{max} = 47°$; 2952 reflections collected of which 2713 were unique; 239 parameters; GOOF = 1.012; $R_1 = 0.0599$, $wR_2 = 0.1103$ for $I > 2\sigma(I)$; residual electron density: 0.215 and -0.161 e $Å^{-3}$. CCDC-186118 (**4**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB21EZ, UK; fax: (+44) 1223-336-033; or deposit@ccdc.cam.ac.uk).
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NMR Reporter Screening for the Detection of High-Affinity Ligands

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The ability to detect protein–ligand interactions in a robust and sensitive manner ("NMR screening") is one of the prime assets of biomolecular NMR spectroscopy in drug discovery.[1–6] It is being used for validation of hits identified in other biological assays, or for the discovery of high-affinity ligands for a given target. NMR screening can be performed by observation of either target resonance signals or ligand resonance signals. Observation of target resonance signals requires large amounts of isotopically labeled protein, very-soluble protein, and has an upper limit for protein size. Since many therapeutically interesting targets do not fall into this class, observation of ligand resonances is the more generally applicable method. A variety of NMR techniques are available to perform these studies. None of them requires isotopically labeled protein, and some of them require only very small amounts of protein.[7–9] A general drawback of all ligand-observation methods, however, is their inability to detect high-affinity ligands—these are false negative and appear to be non-binding. The cause of this significant drawback lies in that for ligand-observation methods, only unbound ligand is observed because of the high excess of ligand over protein. In the case of weak or medium binding affinity (K_D typically less than 1 μM), the dissociation rate is generally so large that a significant exchange between bound and free ligand occurs during the time scale of the experiment, typically a few hundred milliseconds. This exchange process transfers ligand properties from the bound state (e.g. fast relaxation, large negative NOEs) to the free ligand, so that information about binding has been conveyed to the unbound ligand. In strongly bound ligands (sub-micromolar K_D), the

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slow dissociation rate prevents the information of binding to be conveyed to the unbound ligand, so that these strongly bound ligands appear false negative.

Herein we present a novel technique, called NMR reporter screening, which enables the detection of high-affinity ligands by observation of ligand resonance signals. The method has a number of additional advantages: It only detects ligands if they bind to the protein active site while not detecting unspecific binding. Only small amounts of protein in natural isotopic abundance are needed, the experimental time for data collection is so short that it is not throughput-limiting, and data acquisition and analysis can be easily automated.

NMR reporter screening changes the principles of NMR screening: Not the binding of test compounds to the protein target is directly observed, but the ability of a test compound to displace a known ligand, which binds to the protein with medium affinity and which is routinely added as a "reporter ligand" to the mixture of protein and test compounds. Figure 1 shows the principle of the experiment: In the presence of target protein but in the absence of test compounds (Figure 1 b), the reporter ligand is bound to the target protein with moderate affinity, and its relaxation rate is increased as a result of fast relaxation in the bound state, and because of intermediate exchange broadening. This leads to moderate or severe line broadening of the reporter ligand resonances. The same situation prevails if test compounds are added that do not bind to the protein target, bind more weakly than the reporter ligand, or bind non-specifically at a different non-competitive binding site: The reporter ligand is still bound to the target, which can easily be seen by its broadened resonances. However, if test compounds are added that bind to the active site more strongly than the reporter ligand (Figure 1 c), the reporter ligand is displaced from the binding site, so that it becomes unbound. Unbound reporter ligand can be readily distinguished from bound receptor ligand by its sharp resonance signals as seen in a simple 1D proton spectrum, or by other NMR observables such as STD[7] or waterLOGSY[8] signal intensity.

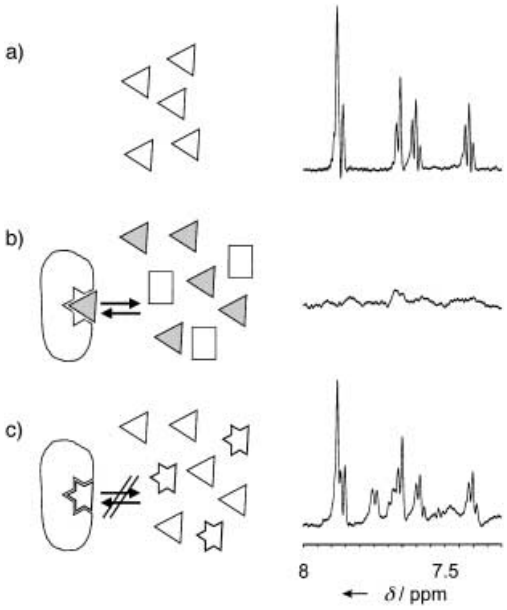

Figure 1. Principle of NMR reporter screening. NMR reporter screening detects the resonance signals of a reporter ligand (triangles) in the presence of target protein and test compounds by simple 1D proton spectra. a) Without protein target, the reporter ligand has sharp signals typical for a small molecule. b) Broad signals of the reporter ligand indicate that the reporter ligand is bound to the target, and that no test compound has comparable binding affinity. c) Sharp signals of the reporter ligand indicate unbound reporter ligand: At least one of the compounds binds more tightly to the protein target and thus displaces the reporter ligand. In the sketch, compounds with increased relaxation (broad lines) are shaded gray, compounds with sharp lines are white. 4 μM spin-labeled 3α-HSD and 100 μM reporter ligand were used for (b) and (c).

The reporter screening technique is illustrated with the example of human 3α-HSD type III, a hydroxysteroid dehydrogenase that is involved in the biosynthesis and degradation of allopregnanolone (5α-pregnan-3α-ol-20-one), a potent positive endogenous allosteric modulator of $GABA_A$-receptor function. Considering the wide neuropsychopharmacological profile of allopregnanolone and other 3α-reduced neuroactive steroids, 3α-HSD type III is a potential target for the treatment of mental disorders such as depression, anxiety, and schizophrenia.[10–12] NMR screening has been applied to identify a small fragment, 2-acetylbenzofuran (**1**), which binds to the active site of 3α-HSD as indicated by the NMR data and by its ability to inhibit the enzymatic activity of the protein. A molecular model of the complex between 3α-HSD and **1** is shown in Figure 2. Compound **1** fits favorably into the binding pocket of 3α-HSD, yet there is space for additional groups to make favorable interactions. The resonance signals of compound **1** experience severe line broadening in the presence of 3α-HSD as a result of increased relaxation in the bound state and intermediate exchange broadening (Figure 1 b).Compound **1** is therefore an ideal reporter ligand since a simple pulse-acquire proton spectrum reveals whether the reporter ligand is bound or unbound. To identify more potent inhibitors of 3α-HSD, 713 candidate ligands from the Novartis compound database were docked to a model of the X-ray structure of 3α-HSD by using the docking program GOLD[13] followed by TAFF[14]/X-PLOR[15] minimization. 30 of the top-ranking compounds were selected by visual inspection for NMR reporter screening. These test compounds were added to a solution of 3α-HSD to which **1** was

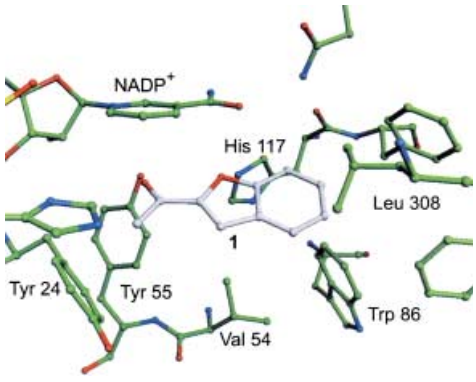

Figure 2. High-scoring model of the complex between human 3α-HSD and **1** obtained by the docking program GOLD, based on an in-house apo crystal structure of human 3α-HSD type III at 2.0 Å resolution.[21] The carbonyl group of **1** (gray skeleton, O atoms in red) is placed below the pyridinium ring of the cofactor $NADP^+$ and the carbonyl and ether O atom of **1** are hydrogen bonded to the hydroxyl group of Tyr 55 and the NE2 atom of His 117, respectively. The main non-polar contacts of **1** are with Tyr 24, Tyr 55, Trp 86, and Leu 308; O red, N blue, C green.

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added as reporter ligand. Screening of these compounds as five mixtures of six compounds took only 30 min experimental time, and identified three potent inhibitors that were able to displace the reporter ligand **1**.

To quantify the displacement of reporter ligand (R) from protein (P) by test compound (C), the ternary system in Equation (1) was considered and the resulting cubic poly-

$$PR + C \overset{K_R}{\rightleftharpoons} P + R + C \overset{K_C}{\rightleftharpoons} PC + R \quad (1)$$

nomial equation describing the concentration of PR was numerically solved.

In Figure 3, the percentage of bound reporter ligand, as measured by NMR spectroscopy, is plotted versus the added concentration of test compound, for three different test

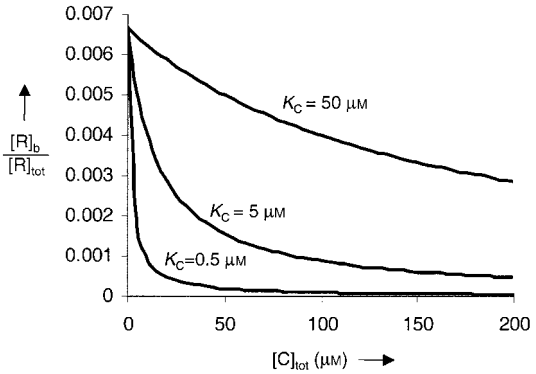

Figure 3. Ratio of bound reporter ligand, $[R]_b$ to $[R]_{tot}$, as a function of total test compound concentration, $[C]_{tot}$, for three different dissociation constants of C between $K_C = 0.5$ μM and $K_C = 50$ μM. The curves have been computed with $[P]_{tot} = 1$ μM (total protein concentration), $[R]_{tot} = 100$ μM (total reporter ligand concentration), $K_R = 50$ μM (dissociation constant of reporter ligand).

compound affinities. To displace 50% of the reporter ligand under typical conditions (1 μM protein concentration, 100 μM reporter ligand concentration, 50 μM reporter ligand affinity), the calculations show that for test compound affinities of 50 μM, 5 μM, and 0.5 μM, test compound concentrations of 150 μM, 15.5 μM, and 2 μM, respectively, are required. Displacement of 50% reporter ligand can be observed by reporter screening, so that these numbers indicate the detection limit of the method. If the percentage of bound reporter ligand is calibrated by NMR spectroscopic parameters, such as relaxation time, line width, or STD signal, the NMR parameter at a given test-compound concentration can be used to calculate the dissociation constant, K_C, of this test compound. Dissociation constants can thus be determined in a one-point measurement, based on the known dissociation constant of the reporter ligand.[16,17]

The sensitivity of NMR reporter screening in terms of protein consumption depends on the relaxation rate enhancement of the reporter ligand in the bound state. This enhancement will generally be largest for ligands that bind in the 1–20 μM K_D range, where relaxation enhancement from intermediate exchange processes can contribute significantly to the overall relaxation rate. The recently introduced SLAPSTIC method[9] can be ideally applied to NMR reporter screening since it drastically increases relaxation in the bound state even in the absence of exchange broadening. In fact, all our NMR experiments on 3α-HSD used spin-labeled 3α-HSD which was readily prepared by the reaction of the lysine side chains with 1-oxyl-2,2,5,5-tetramethylpyrroline-3-carboxylate *N*-hydroxysuccinimide ester (Toronto Research Chemicals, Inc.). The preparation of spin-labeled 3α-HSD required only 4 hours of work time and overnight reaction time. Mass spectrometric analysis indicated that on average, 15 out of the 26 lysine side chains were modified. Control experiments were carried out also with non-spin-labeled 3α-HSD. Spin labeling reduced the protein amounts required for NMR reporter screening by about a factor of 15 for 3α-HSD. With reporter ligand **1**, spin-labeled 3α-HSD concentrations of 4 μM yield drastic effects (see Figure 1b), while the effects with 200 nM spin-labeled 3α-HSD are still sufficiently strong if T1ρ-edited spectra[18,19] are recorded.

If mixtures consisting of a large number of compounds are to be screened in the presence of reporter ligand, resonance overlap of the test compounds with the reporter ligand can make data analysis difficult. In this case, it may be advisable to introduce an isotope label into the reporter ligand, and to record isotope-edited 1D proton spectra, which do not suffer from signal overlap with the (unlabeled) test compounds. Reporter ligand **1** with a ^{13}C-acetyl methyl group was prepared in two steps from coumarilic acid by Grignard addition of $^{13}CH_3MgI$ to the corresponding Weinreb amide. Figure 4 shows examples of homonuclear spectra using unlabeled **1** and isotope-edited spectra using ^{13}C-labeled **1**. While the spectra with unlabeled **1** are hardly interpretable because of severe signal overlap, analysis is much easier with isotope-edited spectra using ^{13}C-**1**.

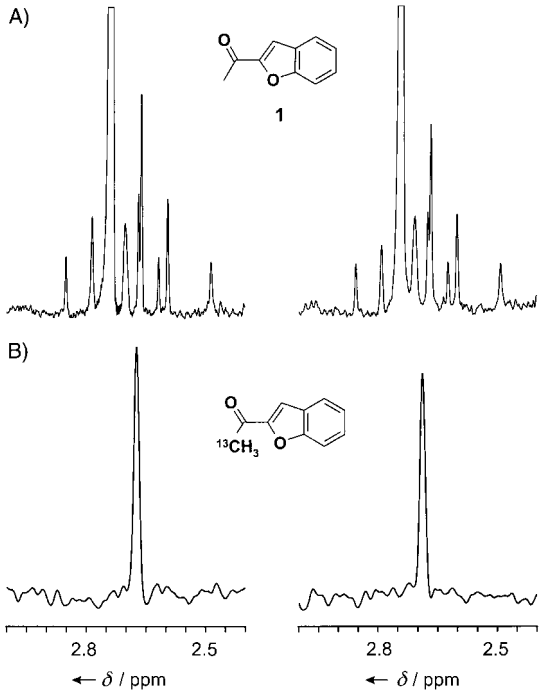

Figure 4. Signal overlap of the reporter ligand with test compounds can be circumvented by isotopic labeling of the reporter ligand. While the test compound signals obscure the relevant signal from T1ρ spectra[18,19] of the unlabeled reporter ligand (A), ^{13}C-edited T1ρ proton spectra of ^{13}C-labeled reporter ligand are devoid of overlap problems (B); 200 nM of spin-labeled 3α-HSD were used. The T1ρ periods were 10 ms (left) and 100 ms (right). All experiments were carried out at 296 K on a Bruker DRX600 instrument.

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Competition assays have been widely used for high-throughput screening in a variety of assay formats, but their use in NMR spectroscopy has so far been limited to investigating specificity of binding, and to measure dissociation constants of known ligands.[16,17,20] NMR reporter screening eliminates the major shortcoming of all current ligand-observation methods of not being able to detect high-affinity ligands with slow dissociation rates. Moreover, it also solves the problem of non-specific binding: Only ligands binding to the active site displace the reporter ligand and appear positive in the reporter screening assay, provided that the reporter ligand binds at the desired binding site. While test compounds need to be fairly soluble (50–500 μM) for current NMR screening methods, NMR reporter screening works even for poorly soluble ligands, if their affinities are high enough, since the test compounds are not actually observed. Finally, NMR reporter screening is very fast since it requires only the acquisition of a simple pulse-acquire spectrum, which typically takes only a few minutes. The throughput of samples is then effectively dominated by the need for sample exchange, temperature equilibration, and magnet shimming, rather than by the duration of the NMR experiment itself. Optionally, the target protein can be spin-labeled to enhance the effect and reduce protein consumption by a factor of 10, and/or the reporter ligand can be isotopically labeled to facilitate spectral interpretation. The reporter ligand does not need to be so-called drug-like. It can be found by NMR screening, or could even be a peptidic ligand. We expect that NMR reporter screening will be of widespread use in NMR-supported drug design.

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[21] Full structural details will be reported elsewhere.

Molecular Encapsulation of Anions in a Neutral Receptor**

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We describe here molecular assemblies that allow the direct observation of individual anions in contact with one or two solvent molecules. The system involves reversible encapsulation of ionic guests in uncharged, synthetic host structures. The anions and solvent are held in confined spaces and extended times amenable to NMR spectroscopy in solution at ambient temperatures. Anions and cations can be even further separated by placement in different capsules.

Recognition of anions by synthetic receptors usually involves charge–charge interactions, hydrogen bonds, or coordination contacts with metals, all carefully arranged in space.[1–5] Reversible encapsulation complexes, in which various anionic guests are surrounded by positively charged hosts, are well-characterized in solution and in the solid state.[6–12] Anions alone are not known to be sequestered in, or even recognized by neutral, reversibly formed capsules but, on occasion, they can be encapsulated as ion pairs.[13–15] We report here the unexpected affinity of neutral cylindrical capsule $\mathbf{1}_2$[16] (the dimeric form of **1**, Figure 1) for anions in $CDCl_3$. The anions are bound reversibly along with one or two solvent molecules. The polar seam of hydrogen-bonded imide groups of $\mathbf{1}_2$ is undoubtedly involved in the recognition process, but even anions not known for hydrogen bonding are readily encapsulated.

The ^{1}H NMR spectrum of cavitand **1** in $CDCl_3$ (Figure 2a) shows the characteristic pattern of the vase[17] conformation but the N–H chemical shift at $\delta = 9.85$ ppm indicates hydrogen bonding typical of a dimeric, capsular structure $\mathbf{1}_2$. Neutral guests such as *trans*-stilbene and benzanilide are readily encapsulated by $\mathbf{1}_2$ in [D_{12}]mesitylene (a solvent too large to fit in the capsule) but not in $CDCl_3$. These guests, at the millimolar concentrations of NMR experiments, cannot compete for the cavity with the solvent at concentrations of about 12 molar. Remarkably, anions can displace the resident solvent. Addition of tetrabutylammonium *p*-toluenesulfonate ($Bu_4N^+TsO^-$) to a solution of $\mathbf{1}_2$ in $CDCl_3$ gave a complex of reduced symmetry, featuring two NH signals and aromatic signals of the resorcinol rings. The resonance signal of the methyl protons of the encapsulated TsO^- ion appeared at $\delta = -2.86$ ppm ($\Delta\delta = -5.1$ ppm), which positions them near the resorcinarene rings of $\mathbf{1}_2$ (Figure 2b). Integration confirmed the stoichiometry of the complex, indicating that the new signals correspond to a complex of $\mathbf{1}_2$ with encapsulated

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