Zuschriften

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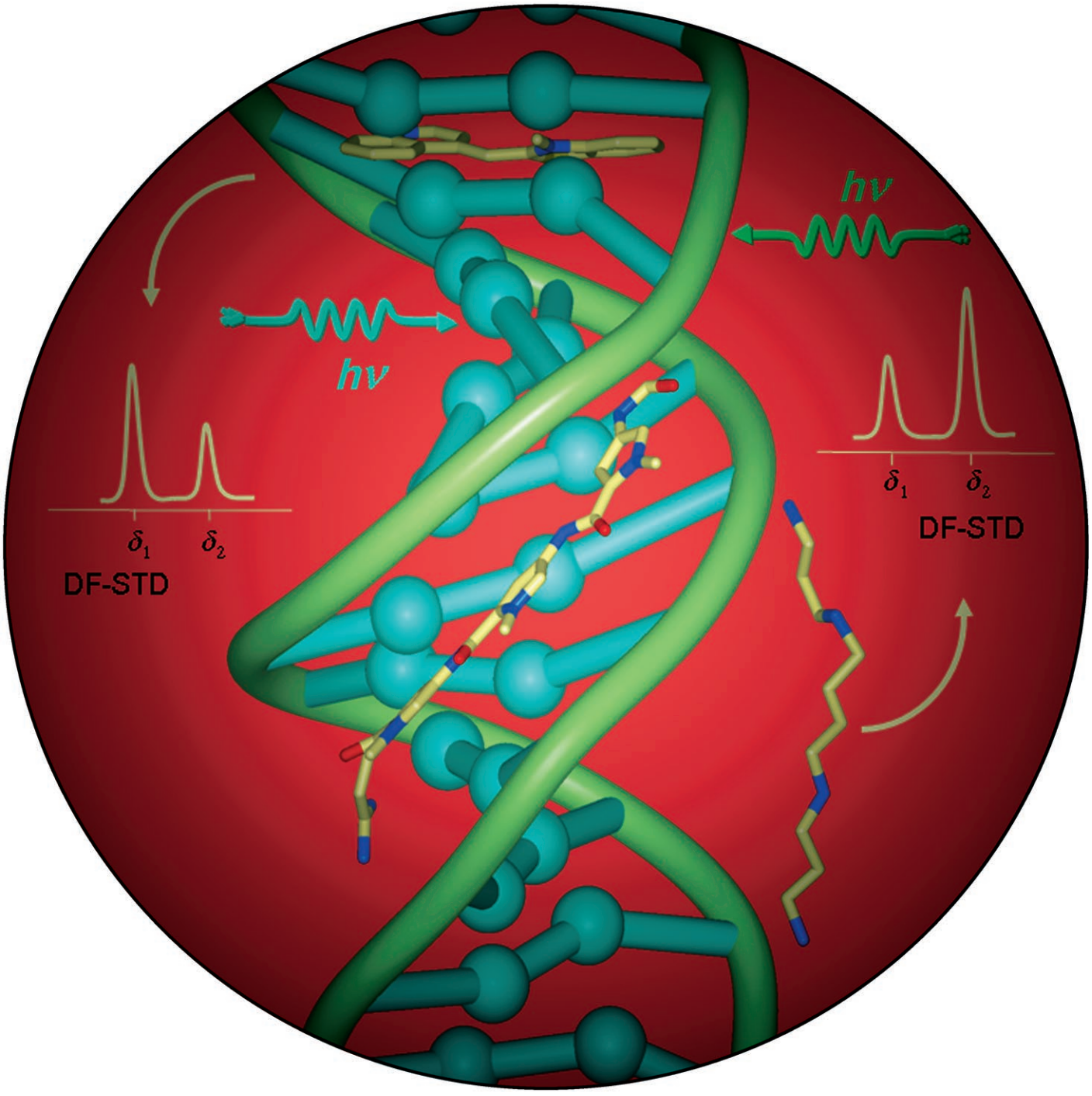

Mit der Differenzialfrequenz-Sättigungstransfer-Differenz(DF-STD)-Spektroskopie, einem neuen NMR-Ansatz zum Screening des Bindens kleiner Moleküle an DNA, hat man eine praktische Sonde für unterschiedliche Arten der Bindung zwischen Liganden und DNA in Händen, wie Gomez-Paloma et al. in ihrer Zuschrift auf den folgenden Seiten beschreiben.

DNA Binding

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Differential-Frequency Saturation Transfer Difference NMR Spectroscopy Allows the Detection of Different Ligand–DNA Binding Modes**

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Ligand-based NMR spectroscopic approaches that allow the convenient probing of ligand–biomolecule binding phenomena have recently gained high visibility in the fields of chemical biology and medicinal chemistry.[1] In particular, saturation transfer difference (STD) spectroscopy[2] offers several advantages: a modest requirement of an unlabeled biological target, virtually without upper limits on its size; amenability to screening mixtures of putative ligands in the presence of non-ligands, molecules that target viruses,[3] and membrane-bound receptors on living cells;[4] and rapid access to relevant information on ligand epitope maps.[5] A main limitation, common to all ligand-based approaches for NMR screening, is that no direct information can be gathered on the intervening receptor surface, and therefore on the mode and site of binding, even if an indirect method (SOS-NMR; SOS: *s*tructural information using *O*verhauser effects and *s*elective labeling) based on selective protein labeling has been devised.[6] Indeed, the high efficiency in diffusing NMR saturation, which is typically observed in globular macromolecules (usually welcomed for the general applicability of STD methods), prevents the observation of any local effect that might be used to monitor the mode and site of binding of the ligand to the receptor surface.

Although such a consideration applies to most biological macromolecules, long tracts of helical DNA may be expected as a notable exception, as they are characterized by a marked difference in their vertical and horizontal dimensions. This effect, in turn, translates into a remarkable anisotropy in the efficiency of saturation diffusion along the two axes. Actually, we were at first surprised to observe important anisotropic effects in saturation diffusion, in the form of different experimental outcomes that depended on the chosen saturation frequency (and therefore DNA region), as such effects are normally not seen in the context of ligand–protein STD spectra.[7] We have found that such an intrinsic feature of STD-NMR experiments employing duplex DNA as receptor allows, at least partly, a differential monitoring of binding events that occur at specific regions of duplex DNA.

Herein, we report a novel STD-based approach that proved particularly valuable for the NMR screening of DNA binders, thus providing key information on their binding mode and allowing the convenient probing of DNA recognition phenomena such as those of base-pair intercalators, minor groove binders, and external backbone binders.[8] The impact of such a technique on drug-discovery processes may be appreciated by taking into account the great importance of small molecules specifically targeting nucleic acids, in the context of anticancer, antifungal, and antiviral drugs. In essence, our approach takes advantage of the difference in STD effect arising from the saturation of protons located in different DNA regions. Data from preliminary STD spectra on ligand–DNA complexes showed that saturation diffusion is far from being isotropic in long tracts of DNA.

The approach described herein is based on two parallel sets of STD experiments performed under the same experimental conditions, in which saturation is elicited by irradiating at a suitable frequency chosen from specific DNA resonances. The signal-to-noise (S/N) ratios of all the protons showing STD effects are measured with reference to an STD spectrum with an off-resonance irradiation. A comparative analysis of such effects in the two sets is then performed. For this reason, we named the method differential-frequency STD (DF-STD) spectroscopy. Besides presenting all the typical advantages of STD spectroscopy, DF-STD spectroscopy has the distinct attribute of providing direct information on the mode of binding of the ligand onto the DNA molecule. To devise our approach, we considered a poly(dG-dC)·poly(dG-dC) copolymer as receptor[9] and the following ligands, each of them characterized by a well-known DNA binding mode: spermine (**1**), a polyamine external binder; distamycin (**2**) and netropsin (**3**), two minor groove binders; and thiazole orange (**4**), doxorubicin (**5**), and ethidium bromide (**6**), three base-pair intercalators.

In particular, the two sets of STD spectra were recorded with saturation by a train of single 50-ms Gaussian pulses, centered either in the aromatic or in the low-field aliphatic spectral regions, where the proton resonances of purine/pyrimidine bases and deoxyribose/backbone,[10] respectively, can be found. This selection reflects the need to propagate saturation to the ligand starting from nuclei lying in different regions of the DNA helix. In this scenario, a ligand making proximate contacts with aromatic base protons, such as an intercalator sandwiched by consecutive base pairs, would receive more saturation upon irradiation of DNA aromatic protons rather than irradiation of deoxyribose protons. Conversely, an external ligand presenting the majority of its binding surface in contact with the DNA phosphodiester backbone should be more affected by irradiation in the deoxyribose region (especially H5′/H5″ in the DNA backbone) rather than by irradiation in the DNA aromatic region.

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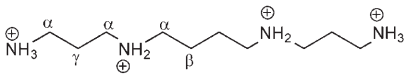
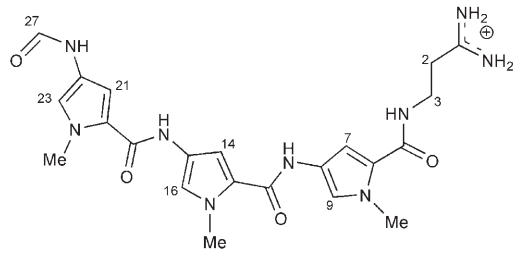
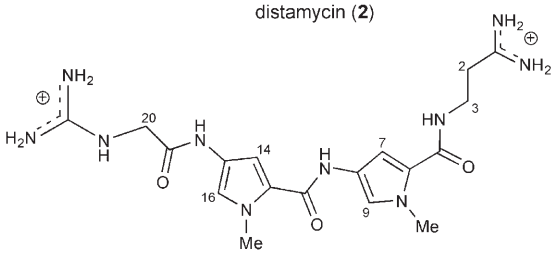
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.



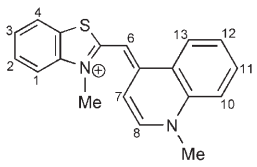
spermine (**1**)



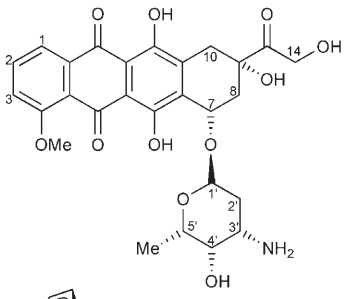
distamycin (**2**)



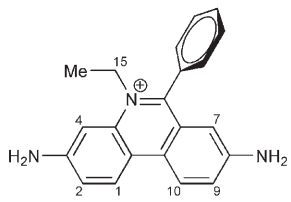
netropsin (**3**)



thiazole orange (**4**)



doxorubicin (**5**)



ethidium bromide (**6**)

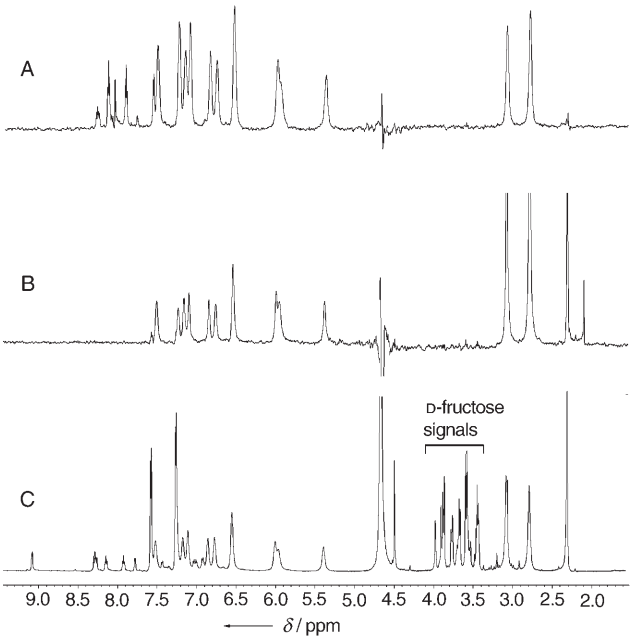


Figure 1. DF-STD spectra of the **4**–DNA complex. A, B) STD spectra recorded upon saturation in the aromatic (8.1 ppm) and deoxyribose/backbone (2.1 ppm) spectral regions, respectively. C) Reference STD spectrum with an off-resonance irradiation. D-Fructose, a non-ligand, was employed as a spy molecule to ensure that only actual STD effects were observed. S/N ratios for all individual proton resonances in both DF-STD spectra can be found in the Supporting Information.

In this respect, minor groove binders are unusual in that they make close contact with both the DNA backbone and base pairs, as the positioning of such ligands, which are often deeply inserted into the DNA minor groove, warrants their proximity to both DNA aromatic and deoxyribose/backbone functionalities. Therefore, for this category of DNA binders we should not expect, at least in principle, a sensitive difference in the outcome of the two sets of STD spectra.

Actually, a comparative analysis of the DF-STD spectra of the six ligands used in the present study confirmed our expectations, to the extent that each ligand with a given DNA binding mode showed a clear-cut DF-STD profile. For instance, Figure 1 shows reference and DF-STD spectra for the **4**–DNA complex. The different response of ligand protons to the two kinds of saturation, and in particular the higher intensity of STD effects upon saturation of DNA base protons, can be appreciated even by visual inspection. This result suggests a close proximity of ligand nuclei to base-pair functionalities, as expected for an intercalative mode of binding. The DF-STD spectral data for all the ligand–DNA complexes examined are reported in Table 1 (for the full data set, see the Supporting Information). To facilitate analysis of the DF-STD data, we propose the introduction of the binding mode index (BMI), a numerical parameter that expresses the relative sensitivity of ligand protons to the perturbation arising from each type of saturation (that is, base versus sugar/

Table 1: BMI values for ligands **1**–**6** computed by using Equation (1).

Ligand	Binding mode	BMI
1	external binder	0.40
2	minor groove binder	1.10
3	minor groove binder	0.90
4	base-pair intercalator	1.40
5	base-pair intercalator	1.33[a]/0.20[b]
6	base-pair intercalator[c]	0.90

[a] BMI calculated for the sole polyaromatic portion (base-pair intercalator). [b] BMI calculated for the sole sugar portion (external binder). For the role of the sugar domain of **5** in DNA binding, see reference [11]. [c] Ethidium bromide is an unusual type of intercalator, which also establishes interactions with the phosphodiester backbone.[12]

backbone saturation). The BMI is computed as given in Equation (1), where $SN_{aromatic}$ and $SN_{aliphatic}$ are the signal-to-

$$BMI = \frac{\sum_i \left(\frac{SN_{aromatic}/SN_{ref}}{SN_{aliphatic}/SN_{ref}} \right)}{n_i} \quad (1)$$

noise ratios relative to STD peaks elicited by saturation of the aromatic bases and deoxyribose/backbone, respectively, SN_{ref} is the signal-to-noise ratio of STD peaks in the reference spectrum, and n_i is the number of ligand peaks considered. On the basis of the data gathered in our tests (see Table 1), we can extrapolate three BMI ranges: $0 < BMI < 0.50$ for external (nonspecific) electrostatic backbone binding; $0.90 < BMI < 1.10$ for minor groove binding; and $1.20\ (0.90) < BMI < 1.50$ for base-pair intercalation.

One point that deserves special comment concerns the BMI values found for base-pair intercalators. In fact, the DF-STD data show that at least two cases of intercalation can be identified. Simple intercalators, such as **4** (BMI = 1.40) or the tricyclic chromophoric portion of **5** (BMI = 1.33), whose interaction with DNA is exclusively based on extended van der Waals contacts with base-pair aromatic rings, can be clearly differentiated from more unusual intercalators. These include ligand **6** (BMI = 0.9), which is also involved in secondary electrostatic interactions with the DNA backbone. The apparent inconvenience of a potential overlap of BMI values between minor groove binders and some intercalators, such as ligand **6** and possibly others, does not seem, in our opinion, a major drawback of the DF-STD approach. Conversely, as DNA intercalators can usually be recognized by their typical structural fingerprint, DF-STD data can add fine details to the big picture of a general base-pair intercalation, the latter often being predicted on the sole basis of the structural features of the ligand. Another interesting case is offered by ligand **5**, which is characterized by two portions that exert two different DNA binding modes. In fact, an intercalative mode of binding is sustained by its tricyclic chromophoric core, and an external electrostatic phosphate binding is attributed to its amino sugar moiety. Again, the DF-STD data, with the two BMI values of 1.33 and 0.20, respectively, seem to describe a pattern perfectly in line with the previously acquired knowledge on **5**–DNA binding characteristics (see Supporting Information).

In conclusion, we have devised a novel STD-based approach that allows the convenient study of small molecule–DNA binding phenomena. We believe that such studies extend in a useful way the application of STD spectroscopy to the realm of chemical biology and medicinal chemistry of nucleic acids.

Experimental Section

STD-NMR experiments were performed on a Bruker Avance DRX 600-MHz spectrometer at 300 K. NMR samples were prepared by dissolving the ligand and the poly(dG-dC)·poly(dG-dC) copolymer in D_2O (500 μL, 99.996%, CIL Laboratories) containing phosphate-buffered saline at pH 7.1. A high ligand–receptor molar excess (20:1) was used for the best STD effects. In particular, the concentration of ligands **1**, **2**, **3**, **5**, and **6** was set at 1.8 mM, whereas that for **4** was 1.5 mM. The concentration of D-fructose was 1 mM. All compounds were purchased from Sigma-Aldrich and used without further purification. The concentration of the double-stranded DNA copolymer (purchased from Pharmacia Biochemicals) was 90 μM, expressed as molarity of phosphate groups.

The STD effects of the individual protons were calculated for each compound relative to a reference spectrum with off-resonance saturation at $\delta = -16$ ppm. Typically, 64 scans were recorded for the reference STD spectrum, whereas 128 scans were recorded for each DF-STD spectrum (saturation time = 4 sec). The relative STD effect was calculated for each signal as the difference between the intensity (expressed as S/N ratio) of one signal in the on-resonance STD spectrum and that of the same signal in the off-resonance NMR spectrum divided by the intensity of the same signal in the off-resonance spectrum. BMI values were obtained by using Equation (1).

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[7] STD-NMR has largely been employed for detecting small molecules interacting with protein targets, whereas very few applications focus on RNA receptors and, to the best of our knowledge, none on DNA systems.

[8] Small molecules binding noncovalently to DNA are usually classified into the cited three main classes: external binders, often polyamines making nonspecific electrostatic contacts with the DNA backbone; minor groove binders, the most sequence-selective class thanks to a network of specific H bonds to base-pair and backbone functions; and base-pair intercalators, which establish extended, and partly specific, van der Waals contacts with the floor of aromatic base pairs.

[9] We also performed test STD experiments on small oligonucleotides. These tests confirmed the importance of having a long

DNA sequence as receptor to give good-quality STD spectra. Accordingly, the DF-STD data were of poor quality in this case. Thus, a short DNA or RNA oligonucleotide (length <20 bp), even if containing a high-affinity binding site for a given ligand, should not be used for STD-NMR experiments.

[10] When saturation within the 4.8–5.5 ppm range, where backbone H5′/H5″ can be found, is prevented by the presence of ligand resonances, irradiation in the 1.5–3.0 ppm range is an alternative that also proved to be similarly valid. We believe that in this case saturation is propagated starting from the resonances of H2′/H2″ of deoxyribose and 5-Me of thymine.

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