Dynamic Chemistry

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Direct STD NMR Identification of β-Galactosidase Inhibitors from a Virtual Dynamic Hemithioacetal System**

*Rémi Caraballo, Hai Dong, João P. Ribeiro, Jesús Jiménez-Barbero, and Olof Ramström**

Dedicated to Professor Jean-Marie Lehn on the occasion of his 70th birthday

Over the last decade, constitutional dynamic chemistry has been successfully demonstrated as a compelling strategy to identify and select new ligands for biological receptors.[1] Key to the formation of dynamic systems are the reversible self-assembly properties of the incorporated system components, allowing rapid rearrangement of all possible associations. Reversible covalent and noncovalent interactions are thus used for component assembly into discrete constituents, resulting in complex systems of an adaptive nature. The exploration of such systems is under rapid development,[2] and the implementation of complex processes are emerging.[3,4] Despite growing interest in the scientific community for dynamic systems, the restricted number of biocompatible, dynamic covalent processes available remains a limitation. An important challenge is therefore the exploration and discovery of efficient reversible reactions and their application to biological systems. In general, dynamic reactions that can be controlled in neutral, aqueous buffer systems are especially advantageous in this context. A further challenge with complex dynamic systems is the analysis of the effects that arise upon interaction with a selector. Therefore, direct in situ analysis of the binding interactions would be a highly convenient means to rapidly identify the appropriate binding partners.[5] These challenges have been addressed in the present study; herein we show that hemithioacetal (HTA) formation is a fast and efficient reversible reaction that can be used to generate systems of potential enzyme inhibitors in aqueous media under neutral conditions. These systems were also subjected to direct binding analysis by saturation transfer difference (STD) NMR spectroscopy, a rapid and efficient technique for protein–ligand binding studies, in which the optimal HTA interactions with the biological target can be readily deduced (Figure 1).

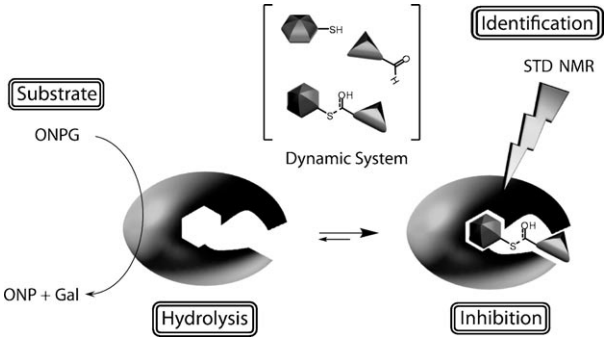

Figure 1. Concept of direct STD NMR identification of enzyme inhibitors from a virtual dynamic hemithioacetal system.

Sulfur-containing components and constituents such as thiols, disulfides, and thioesters occupy a privileged position in aqueous-phase dynamic chemistry owing to their good biocompatibility and their reactivities in reversible processes.[6] However, HTA formation has not yet been applied toward constitutional dynamic chemistry protocols, although these products offer all the requirements stated. The addition of a thiol derivative to a given aldehyde or ketone thus leads to the fast and reversible formation of two HTA stereoisomers, albeit thermodynamically displaced toward the starting components.[7] As a consequence, only a transient amount of HTA is present in solution, resulting in virtual dynamic systems.[8]

To probe hemithioacetals for their performance in dynamic chemistry, a prototype system was designed and tested for binding to a biological receptor. The effects were first evaluated by STD NMR experiments designed to observe and identify the best component associations, and subsequently in separate inhibition studies for validation of the STD NMR results. Thus, the reactions between two aldehydes and five thiol derivatives in water resulted in the spontaneous formation of 10 transient HTA products (20 stereoisomers) in equilibrium with the initial building blocks and two hydrate derivatives (Scheme 1). The system size was, in this case, composed of 29 discrete compounds, all continuously communicating with each other in the reaction vessel. Potential formation of disulfide product was avoided by the use of freshly prepared solutions. The resulting dynamic system was subsequently directed at a target protein; to prevent any competing HTA formation between the aldehydes and a cysteine residue in the active site,[9] β-galactosidase, free of cysteine residues in the active site, was chosen.[10] β-Galactosidase is a well-studied hydrolase that

[*] R. Caraballo,[+] Dr. H. Dong,[+] Prof. O. Ramström
Department of Chemistry, KTH—Royal Institute of Technology
Teknikringen 30, 10044 Stockholm (Sweden)
Fax: (+46) 8-791-2333
E-mail: ramstrom@kth.se
J. P. Ribeiro, Prof. J. Jiménez-Barbero
Centro de Investigaciones Biológicas
Ramiro de Maetzu 9, laboratorio 000, 28040 Madrid (Spain)
[+] These authors contributed equally to this work.

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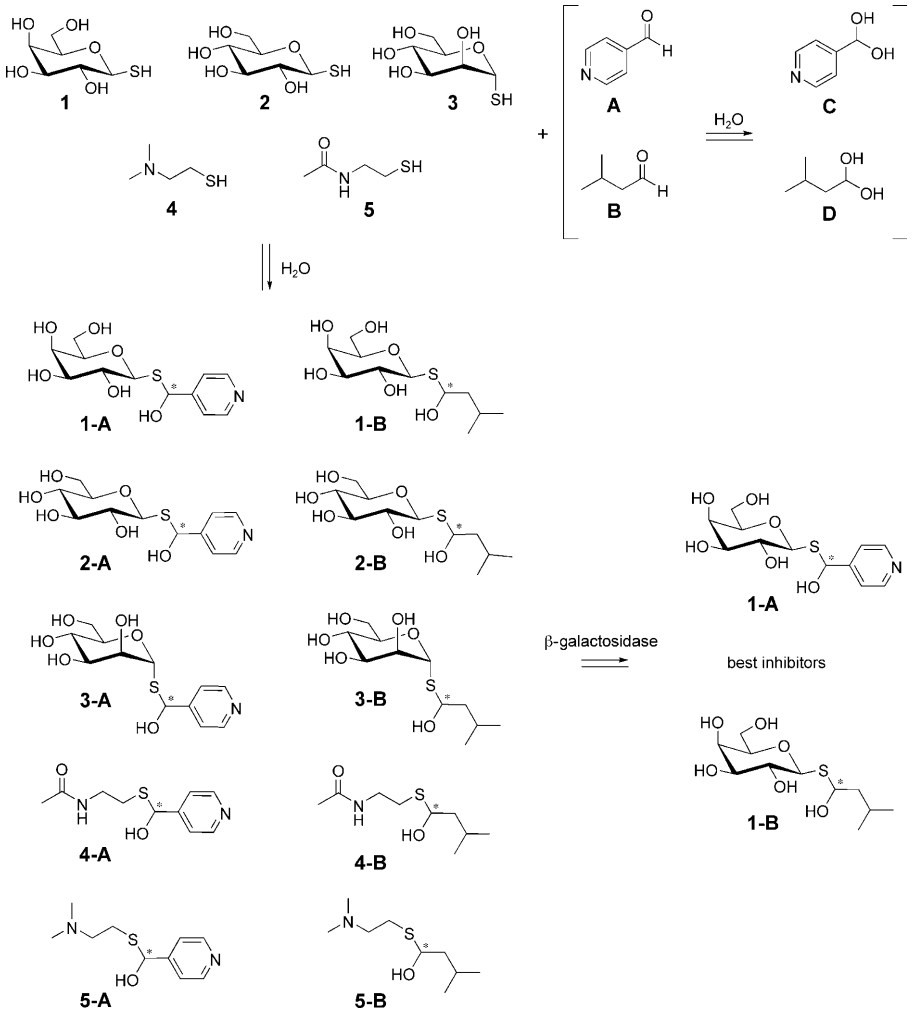

Scheme 1. Virtual dynamic hemithioacetal system formation and enzymatic selection of the best inhibitors.

catalyzes the hydrolysis of the *O*-glycosidic linkage of β-galactosides into their corresponding alcohol and galactose derivatives.[11] The thiol building blocks of the present dynamic system consisted of three 1-thioglycopyranoses (1-thio-β-D-galactopyranose **1**, 1-thio-β-D-glucopyranose **2**, and 1-thio-α-D-mannopyranose **3**) and two alkyl thiol derivatives (2-(dimethylamino)ethanethiol **4** and *N*-acetylcystamine **5**). As electrophiles, aromatic and branched aliphatic aldehydes (pyridine-4-carboxaldehyde **A** and isovaleraldehyde **B**) were chosen because of their similarities to known substrates (*o*-nitrophenyl-β-galactopyranoside (ONPG)) and inhibitors (isopropyl-1-thio-β-D-galactopyranoside (IPTG)) of β-galactosidase, respectively.[12]

Under conditions suitable for the enzyme (pH 7.2, ambient temperature),[13] the HTA formation and dissociation processes are much faster than the NMR relaxation time scale.[7] In our system, the addition of an aldehyde to a thiol in the absence of enzyme did not result in the generation of a characteristic HTA signal;[14] instead, and at a high concentration only (20 mM), spontaneous broadening of the thiol and aldehyde signals occurred. This is consistent with an exchange process and with an equilibrium in favor of the building blocks under these conditions. However, changing the pH from 7.2 to 4.0 considerably decreased the elimination rate and resulted in the formation of specific HTA signals slightly downfield from the hydrate signals of the corresponding aldehydes. However, neutral conditions were used in subsequent studies with the enzyme, in which the latent presence of HTA products, a direct consequence of the very rapid formation/dissociation rate and the thermodynamic stabilities, resulted in virtual dynamic systems.

STD NMR was subsequently adopted for binding studies of the virtual systems with β-galactosidase. The advantage of this spectroscopic technique rests in the exclusive observation of bound ligands to the receptor.[15] Only a small amount of non-isotope-labeled protein (ca. 100 nmol) is required for STD NMR experiments. In these experiments, the magnetization is transferred from the protein protons to those of the bound ligand, which is detected after dissociation from the receptor, provided the dissociation rate is fast relative to the relaxation time scale. Subtraction of the reference spectrum (*off* resonance) from the saturation spectrum (*on* resonance) yields the STD NMR spectrum. However, the distinction between specific and nonspecific binding may be somewhat challenging.[16] It was therefore expected that the aromatic species would produce non-negligible binding due to the presence of hydrophobic regions of β-galactosidase, whereas the glycopyranose moieties were expected to provide only specific interactions. The ^{1}H STD NMR analyses of the overall system, however, completely support the hypothesis that the galactose-containing species would be exclusively selected by the enzyme (Figure 2a,b). The results proved highly conspicuous, and the complex ^{1}H NMR spectra (*off* resonance) were much better resolved in the STD spectra. Among the carbohydrate species, the proton signals from component **1** could be clearly identified in the STD spectra, whereas no signals from components **2** and **3** were thus recorded, attesting to the specific recognition of β-galactosidase by galactose analogues. In addition, insignificant traces of components **4** and **5** were observed. The presence of aldehyde **A**, its corresponding hydrate **C**, and to a lower extent, components **B** and **D** were likely the consequence of simultaneous specific and nonspecific binding (Figure 2b,c). Although the HTA product signals were not distinctly

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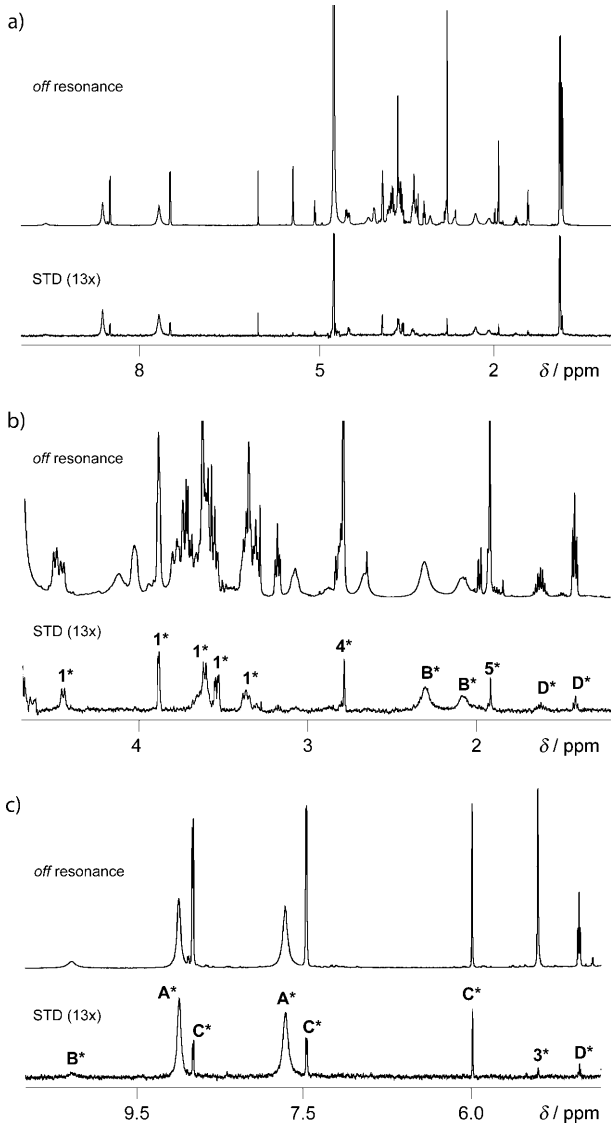

Figure 2. [1]H STD NMR studies: a) full system spectra; enlarged areas of b) the aliphatic region and c) the aromatic region. * Signals from corresponding constituents.

observed from the starting components during the STD NMR experiments, binding of constituents **1-A** and **1-B** (20 μM) to the β-galactosidase active site was recorded. Thus, significant signal broadening of both aldehyde and 1-thio-β-D-galactopyranoside resonance signals, as well as a downfield shift of the anomeric proton (H-1) of **1** were observed in the regular 1D and STD NMR spectra only in the presence of the enzyme (see Figures S6 and S15 in the Supporting Information). Nevertheless, to confirm the structural nature of the enzyme-selected molecules (thiol, aldehyde, and/or HTA), additional [1]H STD NMR experiments were performed. Thus, comparison of the STD spectra of the individual components **1**, **A**, and **B** with the enzyme indicated the presence of interactions between the isolated components and β-galactosidase. However, no broadening of the resonance signals of these molecules was observed in the regular 1D NMR spectra, indicating that the affinity for the isolated components was much lower than that for the HTA constituents **1-A** and **1-B**. In addition, careful examination of the data from the binding experiments provided indications on the binding mode of the carbohydrate moiety inside the active site of the enzyme. Analysis of the STD signal ratios suggested that the C2 and C3 region of the carbohydrate ring is the binding epitope (100 and 80% of the relative STD for H-2 and H-3, respectively; larger than for the other protons). As expected, [1]H STD NMR studies of reduced systems, containing component **1** with either one or both of aldehydes **A** and **B**, resulted in the same observation as with the full system. Nevertheless, modifications of the STD epitope ratios for the galactose moiety in component **1** alone, **1-A** and **1-B**, suggested a different spatial orientation of the three galactose derivatives within the active site. The STD ratios on the C5/C6 region are markedly different for **1-A** and **1-B** (see Figure S14, Supporting Information). The subtle structural changes of the galactose interaction would naturally affect the HTA binding properties, and the specific binding to β-galactosidase would be reinforced or weakened at some positions for the same component. The hypothesized nonspecificity of the binding originating from **A**, **B**, and their corresponding hydrates (**C** and **D**), in the absence of thiol, was also confirmed by competition STD NMR experiments with IPTG at various concentrations (from 50 to 350 equiv). Effectively, the STD signals from **A**, **B**, **C**, and **D** remained constant throughout the titration with IPTG, thus demonstrating their binding to a different site of the enzyme (see Figures S16 and S17, Supporting Information).

To corroborate the [1]H STD NMR results, inhibition studies were performed for each system. ONPG was chosen as substrate, and its enzymatic hydrolysis to *o*-nitrophenol (ONP) and D-galactose could be followed in the presence or absence of the dynamic system components. To ensure uniformity of the results, the same conditions were applied for both binding and inhibition studies, and [1]H NMR spectroscopy was therefore used to measure the formation of ONP over time. The inhibition studies were performed using an inhibitor/substrate ratio of 5:1, and the inhibitory effects were estimated as the ratio of the t_{50} values (time required for 50% ONPG hydrolysis) in the presence and absence of inhibitor. The results clearly support those of the binding studies and thus, as deduced from analysis of the STD experiments, all the thiogalactose-containing derivatives indeed inhibit β-galactosidase activity (Figure 3). Constituents **1-A**, **1-B**, and, to a lesser extent, component **1** showed the strongest inhibition behavior, and the rates of ONPG hydrolysis were approximately 12-, 4-, and 2-fold lower, respectively, relative to the blank reaction. Constituent **2-B** was also found to show trace properties, and exhibited an inhibition value of about 1.5. Interestingly, the enzyme activity was totally unaffected by the presence of the aldehydes and their corresponding hydrates. In addition, none of the other adducts caused any decrease in the rate of ONPG hydrolysis. As a consequence of the virtual character of the system, other interactions between the active species and the enzyme could, in principle, be involved during the process of ONPG hydrolysis. Therefore, two additional

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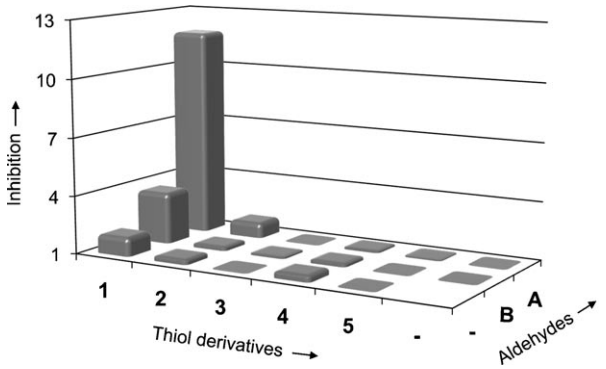

Figure 3. Inhibition studies of the system components and constituents. Inhibition $= t_{50\,(\mathrm{Inh})}/t_{50\,(\mathrm{blank})}$, for which t_{50} = time required for 50% ONPG hydrolysis.

controls involving D-galactose and 4-pyridinylmethanol, the reduced form of aldehyde **A**, were performed to further support the conclusions. Neither of these compounds results in HTA formation, and significant inhibitory effects would indicate other factors of importance. However, no inhibition was observed when D-galactose was tested together with aldehyde **A**, and when 1-thio-β-D-galactopyranose (**1**) was evaluated with 4-pyridinylmethanol, the same trace inhibitory effect as for compound **1** alone was found. Furthermore, and in both cases, no signal broadening was recorded in the regular 1D NMR spectra in the presence of β-galactosidase. This demonstrates that the combination **1-A** is necessary for inhibition.

The combined results from the inhibition studies and the ^{1}H STD NMR experiments unambiguously confirms the formation of virtual HTA systems of β-galactosidase inhibitors. The transient constituents **1-A** and **1-B** were efficiently identified as the best species selected by the enzyme from a total of 19 compounds (HTA stereochemistry not taken into consideration). Furthermore, the lack of effect by the aldehydes and their respective hydrates toward ONPG hydrolysis supports the nonspecific binding character of these entities with β-galactosidase.

In conclusion, we have successfully demonstrated, for the first time, hemithioacetal formation applied to dynamic combinatorial system generation in aqueous media. Equilibrium formation using this chemistry proved very rapid and resulted in truly virtual dynamic systems in which ^{1}H NMR analyses confirmed the presence of transient HTA constituents. It has further been demonstrated that ^{1}H STD NMR spectroscopy can be used for efficient and direct in situ identification of the best enzyme binders of the virtual dynamic system. Inhibition studies unequivocally support the ^{1}H STD NMR experimental data and enable the distinction of different β-galactosidase inhibitors and nonspecific binders. The HTA resulting from the addition of 1-thio-β-D-galactose and pyridine-4-carboxaldehyde proved to be the best inhibitor in the system tested. This combination of dynamic system formation with STD NMR spectroscopy, resolving complex systems by the identification of the best ligands, can be easily expanded to accommodate a wider variety of components, resulting in efficient and rapid mapping of enzyme inhibitory potentials.

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[1] For representative reviews, see: a) J.-M. Lehn, *Chem. Soc. Rev.* **2007**, *36*, 151 – 160; b) B. de Bruin, P. Hauwert, J. N. H. Reek, *Angew. Chem.* **2006**, *118*, 2726 – 2729; *Angew. Chem. Int. Ed.* **2006**, *45*, 2660 – 2663; c) P. T. Corbett, J. Leclaire, L. Vial, K. R. West, J. L. Wietor, J. K. M. Sanders, S. Otto, *Chem. Rev.* **2006**, *106*, 3652 – 3711; d) M. Crego-Calama, D. N. Reinhoudt, M. G. J. ten Cate, *Top. Curr. Chem.* **2005**, *249*, 285 – 316; e) O. Ramström, J. M. Lehn, *Nat. Rev. Drug Discovery* **2002**, *1*, 26 – 36; f) O. Ramström, T. Bunyapaiboonsri, S. Lohmann, J.-M. Lehn, *Biochim. Biophys. Acta Biomembr.* **2002**, *1572*, 178 – 186.

[2] For recent reports, see: a) M. Barboiu, F. Dumitru, Y. Legrand, E. Petit, A. van der Lee, *Chem. Commun.* **2009**, 2192 – 2194; b) Y. Ruff, J.-M. Lehn, *Angew. Chem.* **2008**, *120*, 3612 – 3615; *Angew. Chem. Int. Ed.* **2008**, *47*, 3556 – 3559; c) S. Xu, N. Giuseppone, *J. Am. Chem. Soc.* **2008**, *130*, 1826 – 1827; d) P. Besenius, P. Cormack, J. Liu, S. Otto, J. K. M. Sanders, D. Sherrington, *Chem. Eur. J.* **2008**, *14*, 9006 – 9019; e) A. Bugaut, K. Jantos, J. Wietor, R. Rodriguez, J. K. M. Sanders, S. Balasubramanian, *Angew. Chem.* **2008**, *120*, 2717 – 2720; *Angew. Chem. Int. Ed.* **2008**, *47*, 2677 – 2680; f) R. Ludlow, S. Otto, *J. Am. Chem. Soc.* **2008**, *130*, 12218 – 12219; g) M. Chung, C. Hebling, J. Jorgenson, K. Severin, S. Lee, M. Gagné, *J. Am. Chem. Soc.* **2008**, *130*, 11819 – 11827; h) P. Gareiss, K. Sobczak, B. McNaughton, P. Palde, C. Thornton, B. Miller, *J. Am. Chem. Soc.* **2008**, *130*, 16254 – 16261; i) J. Sadownik, D. Philp, *Angew. Chem.* **2008**, *120*, 10113 – 10118; *Angew. Chem. Int. Ed.* **2008**, *47*, 9965 – 9970; j) R. Caraballo, M. Rahm, P. Vongvilai, T. Brinck, O. Ramström, *Chem. Commun.* **2008**, 6603 – 6605.

[3] a) M. Angelin, P. Vongvilai, A. Fischer, O. Ramström, *Chem. Commun.* **2008**, 768 – 770; b) P. Vongvilai, M. Angelin, R. Larsson, O. Ramström, *Angew. Chem.* **2007**, *119*, 966 – 968; *Angew. Chem. Int. Ed.* **2007**, *46*, 948 – 950; c) R. J. Sarma, S. Otto, J. R. Nitschke, *Chem. Eur. J.* **2007**, *13*, 9542 – 9546; d) Y. M. Legrand, A. van der Lee, M. Barboiu, *Inorg. Chem.* **2007**, *46*, 9540 – 9547; e) V. Goral, M. I. Nelen, A. V. Eliseev, J. M. Lehn, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 1347 – 1352.

[4] a) R. F. Ludlow, S. Otto, *Chem. Soc. Rev.* **2008**, *37*, 101 – 108; b) D. J. Hayden, G. von Kiedrowski, N. Lehman, *Angew. Chem.* **2008**, *120*, 8552 – 8556; *Angew. Chem. Int. Ed.* **2008**, *47*, 8424 – 8428; c) J. Stankiewicz, L. H. Eckardt, *Angew. Chem.* **2006**, *118*, 350 – 352; *Angew. Chem. Int. Ed.* **2006**, *45*, 342 – 344.

[5] a) D. C. Rees, M. Congreve, C. W. Murray, R. Carr, *Nat. Rev. Drug Discovery* **2004**, *3*, 660 – 672; b) M. S. Congreve, D. J. Davis, L. Devine, C. Granata, M. O'Reilly, P. G. Wyatt, H. Jhoti, *Angew. Chem.* **2003**, *115*, 4617 – 4620; *Angew. Chem. Int. Ed.* **2003**, *42*, 4479 – 4482.

[6] a) H. Hioki, W. C. Still, *J. Org. Chem.* **1998**, *63*, 904 – 905; b) O. Ramström, J.-M. Lehn, *ChemBioChem* **2000**, *1*, 41 – 47; c) S. Otto, R. L. E. Furlan, J. K. M. Sanders, *J. Am. Chem. Soc.* **2000**, *122*, 12063 – 12064; d) Y. Krishnan-Ghosh, S. Balasubramanian, *Angew. Chem.* **2003**, *115*, 2221 – 2223; *Angew. Chem. Int. Ed.* **2003**, *42*, 2171 – 2173; e) R. Larsson, O. Ramström, *Eur. J. Org. Chem.* **2005**, 285 – 291; f) B. Shi, M. F. Greaney, *Chem. Commun.* **2005**, 886 – 888; g) R. Larsson, Z. Pei, O. Ramström, *Angew.*

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Chem. **2004**, *116*, 3802 – 3804; *Angew. Chem. Int. Ed.* **2004**, *43*, 3716 – 3718.
[7] a) G. E. Lienhard, W. P. Jencks, *J. Am. Chem. Soc.* **1966**, *88*, 3982 – 3995; b) R. E. Barnett, W. P. Jenks, *J. Am. Chem. Soc.* **1967**, *89*, 5963 – 5964; c) R. E. Barnett, W. P. Jenks, *J. Am. Chem. Soc.* **1969**, *91*, 6758 – 6765; d) H. F. Gilbert, W. P. Jenks, *J. Am. Chem. Soc.* **1977**, *99*, 7931 – 7947; e) H. Fu, J. Park, D. Pei, *Biochemistry* **2002**, *41*, 10700 – 10709.
[8] a) I. Huc, J. M. Lehn, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 2106 – 2110; b) J. M. Lehn, *Chem. Eur. J.* **1999**, *5*, 2455 – 2463.
[9] a) J. O. Westerik, R. Wolfenden, *J. Biol. Chem.* **1972**, *247*, 8195 – 8197; b) M. R. Bendall, I. L. Cartwright, P. I. Clark, G. Lowe, D. Nurse, *Eur. J. Biochem.* **1977**, *79*, 201 – 209; c) C. A. Lewis, Jr., R. Wolfenden, *Biochemistry* **1977**, *16*, 4890 – 4895.
[10] A. V. Fowler, I. Zabin, *J. Biol. Chem.* **1970**, *245*, 5032 – 5041.
[11] a) T. M. Stokes, I. B. Wilson, *Biochemistry* **1972**, *11*, 1061 – 1064; b) R. E. Huber, M. T. Gaunt, *Biochem. Biophys.* **1983**, *220*, 263 – 271.
[12] a) C. K. De Bruyne, M. Yde, *Carbohydr. Res.* **1977**, *56*, 153 – 164; b) H. Sandermann, Jr., *Eur. J. Biochem.* **1977**, *80*, 507 – 515; c) P. J. Deschavanne, O. M. Viratelle, J. M. Yon, *J. Biol. Chem.* **1978**, *253*, 833 – 837.
[13] M. Decleire, N. van Huyhn, J. C. Motte, *Appl. Microbiol.* **1985**, *21*, 103 – 107.
[14] C. Rae, S. I. O'Donoghue, W. A. Bubb, P. W. Kuchel, *Biochemistry* **1994**, *33*, 3548 – 3559.
[15] a) S. Mari, D. Serrano-Gomez, F. J. Cañada, A. L. Corbi, J. Jiménez-Barbero, *Angew. Chem.* **2005**, *117*, 300 – 302; *Angew. Chem. Int. Ed.* **2005**, *44*, 296 – 298; b) B. Claasen, M. Axmann, R. Meinecke, B. Meyer, *J. Am. Chem. Soc.* **2005**, *127*, 916 – 919; c) S. Di Micco, C. Bassarello, G. Bifulco, R. Riccio, L. Gomez-Paloma, *Angew. Chem.* **2006**, *118*, 230 – 234; *Angew. Chem. Int. Ed.* **2006**, *45*, 224 – 228; d) T. Diercks, J. P. Ribeiro, F. J. Cañada, S. André, J. Jiménez-Barbero, H.-J. Gabius, *Chem. Eur. J.* **2009**, *15*, 5666 – 5668; e) B. Meyer, T. Peters, *Angew. Chem.* **2003**, *115*, 890 – 918; *Angew. Chem. Int. Ed.* **2003**, *42*, 864 – 890.
[16] a) J. Liu, D. Begely, D. Mitchell, C. Verlinde, G. Varani, E. Fan, *Chem. Biol. Drug Des.* **2008**, *71*, 408 – 419; b) D. Middleton, *Mod. Magn. Reson.* **2006**, 319 – 326; c) Z. Ji, Z. Yao, M. Liu, *Anal. Biochem.* **2009**, *385*, 380 – 382.

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