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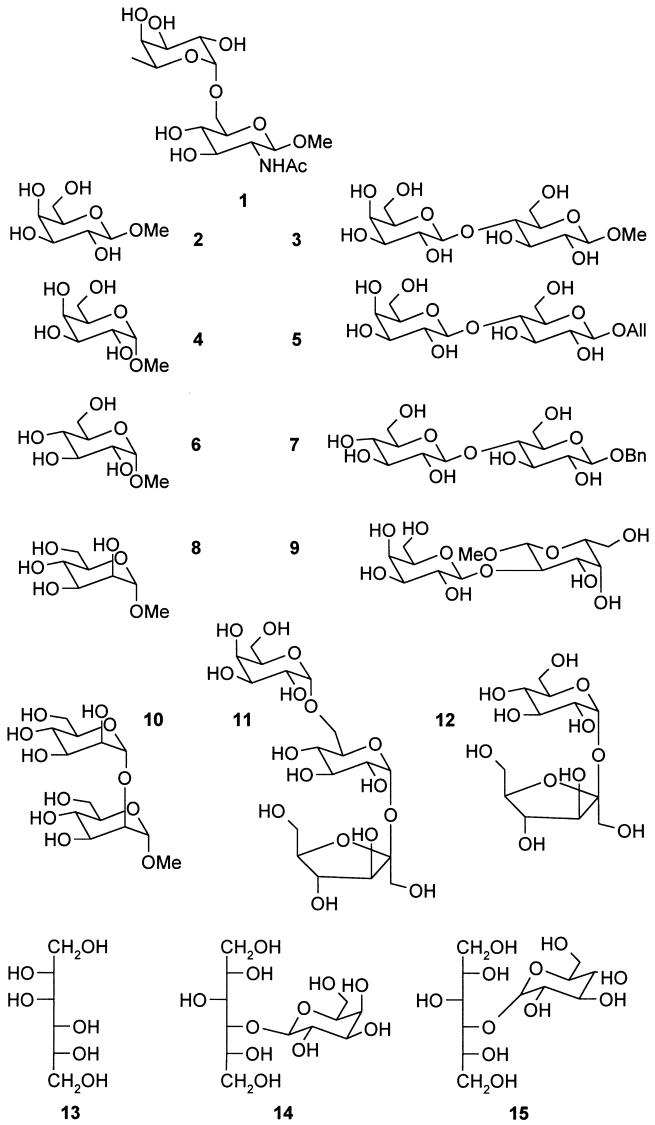
Application of 3D-TOCSY-trNOESY for the Assignment of Bioactive Ligands from Mixtures**

Lars Herfurth, Thomas Weimar, and Thomas Peters*

An increasing body of work demonstrates that NMR spectroscopy provides powerful protocols to screen compound mixtures such as combinatorial libraries for their binding activity towards receptor proteins.[1–5] Compared to other screening techniques, NMR has specific advantages. One important feature is that NMR experiments allow parallel screening protocols without the need to subsequently separate the library components. In addition, NMR potentially delivers precise structural and topological information on the bioactive ligand, the protein binding site, or both. A comparison of NMR screening protocols published so far shows that two main classes of experiments may be distinguished. One category of experiments targets primarily the receptor protein and aims at identifying those amino acids involved in binding. For instance, specific absorption rate measurements, "SAR by NMR", employs ^{15}N labeling of the proteins to allow for fast heteronuclear single quantum coherence (HSQC) experiments.[2] Another class of experiments relies on changes of specific ligand properties upon binding to a receptor protein. In the main, altered relaxation, diffusion, or both have been utilized as the basis for such experiments.[3] It has been recently shown that saturation transfer difference (STD) experiments are especially useful for identifying ligands with binding activity and, furthermore, deliver information on the binding epitope.[4]

Herein, we report on a library consisting of 15 individual carbohydrates (Scheme 1) in the presence of the lectin *Aleuria aurantia* agglutinin (AAA). We have already shown



Scheme 1. Components of the library tested for the binding activity towards AAA. All components are present in approximately 10 mM concentration with a binding site:ligand ratio of 1:20 for each. Details of the sample preparation have been described previously.[5a]

that transfer NOE spectroscopy (trNOESY) experiments deliver typical cross-peak patterns that allow assignment of the bioactive component if all individual compounds are known.[5a] However, without this prior knowledge, an unambiguous assignment would be impossible. In the following, we present a strategy that closes this gap.

[*] Prof. Dr. T. Peters, Dipl.-Chem. L. Herfurth, Dr. T. Weimar
Institut für Chemie der Medizinischen Universität zu Lübeck
Ratzeburger Allee 160, 23538 Lübeck (Germany)
Fax: (+49) 451-500-4241
E-mail: thomas.peters@chemie.mu-luebeck.de

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Initially, a one-dimensional (1D) STD spectrum and a STD-TOCSY spectrum of the library in the presence of AAA were acquired (Figure 1; the 1D STD spectrum is not shown). Two individual spin systems were unambiguously identified. In comparison to trNOESY spectra, STD-TOCSY spectra have

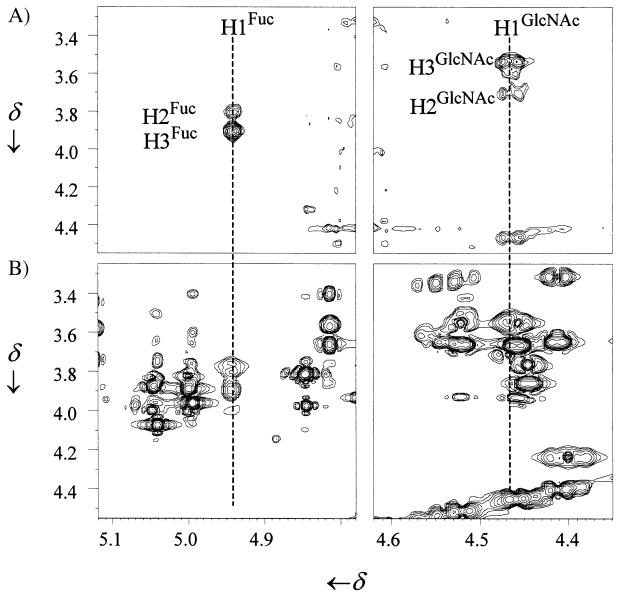
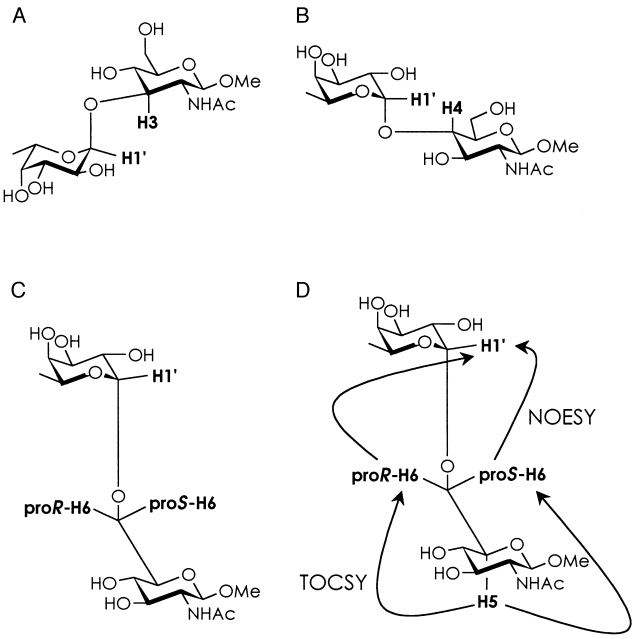


Figure 1. Comparison the region of anomeric protons $H1^{Fuc}$ (left) and $H1^{GlcNAc}$ (right) of a TOCSY (B) and a STD-TOCSY (A) spectrum of the library in the presence of AAA. In (B), only the cross peaks of compound **1** involved in binding remain.

the advantage that cancellation between negative and positive cross peaks does not occur. From the chemical shifts and coupling constants, it was easy to assign the two STD-TOCSY cross peak patterns to an α-L-fucose (Fuc) and a β-D-*N*-acetyl glucosamine (GlcNAc) residue. Important spectral characteristics comprised the C6-bound methyl group of Fuc, the *O*-methyl group, the *N*-acetyl group, and the anomeric protons. From previous trNOESY experiments, it was also clear that the GlcNAc residue was linked to an *O*-methyl group at the anomeric carbon atom. Only the linkage type between the Fuc and GlcNAc remained unknown. From a STD-TOCSY spectrum this information could not be obtained because scalar couplings between protons across glycosidic linkages are much too small to allow for an assignment of the linkage site. Fortunately, interglycosidic NOEs, or trNOEs, contain this information (Scheme 2). In principle, interglycosidic trNOEs are obtained from trNOESY spectra but, in this case, severe overlap between protons of the Fuc and the GlcNAc residue prohibited the discrimination of inter- and intraglycosidic trNOEs. For instance, the $H2^{Fuc}$ signal overlapped with that of pro*S*-$H6^{GlcNAc}$, and the $H3^{Fuc}$ signal overlapped with pro*R*-$H6^{GlcNAc}$, which prevented an unambiguous assignment of the trNOEs to $H1^{Fuc}$. It is well known that resonance overlap can be overcome by increasing the number of dimensions in a NMR experiment. Therefore, a 3D-TOCSY-trNOESY experiment[6] was performed. In this experiment, protons are labeled according to their association



Scheme 2. Interglycosidic trNOESY pathways as a function of the glycosidic linkage type between Fuc and GlcNAc. Predominant interglycosidic NOEs are in bold face. A) (1→3) linkage. B) (1→4) linkage. C) (1→6) linkage. D) Combination of TOCSY and trNOESY pathways allows an unequivocal identification of the linkage site. Magnetization pathways are labeled as arrows and correspond to cross peaks in Figure 2.

with a particular spin system during the TOCSY step and the trNOESY step conveys the necessary interglycosidic trNOE information.

Inspection of the 3D TOCSY-trNOESY spectrum of the library (Scheme 1) in the presence of AAA showed that $H1^{Fuc}$ gave trNOEs to protons that had scalar coupling partners in the spectral region around $\delta \approx 3.5$. From the chemical shifts, it was clear that these protons did not belong to Fuc because this residue had no protons with $\delta < 3.6$, except for the C6-bound methyl group. In addition, two of the protons that gave trNOEs to $H1^{Fuc}$ were scalar coupled to each other. This information was sufficient to identify the linkage type between Fuc and GlcNAc. Figure 2 shows a ω_1/ω_2 slice at the frequency of $H1^{Fuc}$ ($\omega_3 = 4.95$). All cross peaks observed in

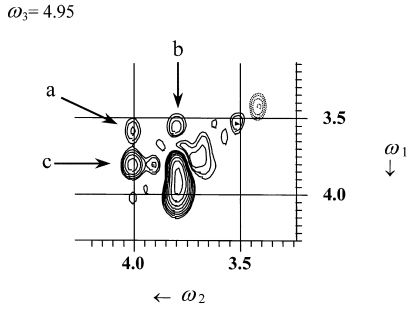


Figure 2. ω_1/ω_2 slice from a 3D-TOCSY-trNOESY experiment, in which ω_3 is set to the resonance frequency of the anomeric proton, $H1^{Fuc}$ ($\delta = 4.95$). Connectivities through scalar coupling (TOCSY transfer, first step) and through space cross relaxation (NOESY transfer, second step) are shown. All cross peaks indicate magnetization that has finally been transferred to $H1^{Fuc}$: a) $H5^{GlcNAc} \rightarrow$pro*S*-$H6^{GlcNAc} \rightarrow H1^{Fuc}$; b) $H5^{GlcNAc} \rightarrow$pro*R*-$H6^{GlcNAc} \rightarrow H1^{Fuc}$; c) pro*R*-$H6^{GlcNAc} \rightarrow$pro*S*-$H6^{GlcNAc} \rightarrow H1^{Fuc}$.

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this slice had corresponding protons that, in common, gave trNOEs to $H1^{Fuc}$. In addition, scalar coupling partners were easily identified since the ω_1/ω_2 slice reflected TOCSY pathways. The scalar coupling pattern was attributed to protons pro*R*-$H6^{GlcNAc}$, pro*S*-$H6^{GlcNAc}$, and $H5^{GlcNAc}$, as indicated in Figure 2. It should be emphasized that the intraglycosidic trNOEs between $H2^{Fuc}$ and $H3^{Fuc}$ were also present in this spectrum and overlapped with the pro*R*-$H6^{GlcNAc}$/pro*S*-$H6^{GlcNAc}$ pattern. However, the scalar coupling of the latter two protons to $H5^{GlcNAc}$ at $\delta = 3.51$ allowed a clear discrimination. Inspection of Scheme 2 shows that only a $(1 \rightarrow 6)$ linkage between the two sugar residues explains the observed cross peak pattern.

In summary, a 3D-TOCSY-trNOESY experiment allowed unambiguous identification of the bioactive component of a carbohydrate library without prior knowledge of the individual components of the library. Carbohydrates certainly represent an important class of biological macromolecules[7, 8] but, nonetheless, the experimental protocol described here is not limited to carbohydrate derivatives.

Experimental Section

All spectra were acquired on a Bruker DRX 500 MHz spectrometer with a 5 mm TXI probehead at 306 K. TOCSY and STD-TOCSY were measured with 512 increments and 16 transients using a 50 ms MLEV-17 spinlock field of 7.5 kHz. Saturation transfer was achieved by using 40 selective 270° Gaussian pulses of duration 50 ms and spacing 10 ms. The protein envelope was irradiated at 2.8 ppm (on-resonance) and 40 ppm (off-resonance). Subsequent subtraction of on- and off-resonance spectra was achieved via phase cycling.

For the 3D-TOCSY-trNOESY experiment, 124, 256, and 1014 data points in F1, F2, and F3, respectively, were acquired with eight transients each. The acquisition time was 127 ms, which results in a total relaxation delay of 1.6 s. The total measurment time was approximately five days. The mixing time for the NOESY step was 130 ms. Suppression of zero-quantum coherence[9] was achieved by parallel application of a spinlock field (2.5 ms, 7.5 kHz) and a gradient pulse (2.5 ms, approximately 5 G cm^{-1}) prior to the NOE mixing time. Signals from HDO were suppressed by presaturation with a weak radio-frequency field.

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Natural-Product Hybrids: Design, Synthesis, and Biological Evaluation of Quinone–Annonaceous Acetogenins**

Sabine Hoppen, Ulrich Emde, Thorsten Friedrich, Lutz Grubert, and Ulrich Koert*

The acetogenins of *Annonaceae* are a class of natural products with interesting antitumor, immunosuppressive, pesticide, and antimicrobial activities.[1] Their main mode of action is the inhibition of the mitochondrial complex I (NADH-ubiquinone oxidoreductase).[2] Mucocin[3] and squamocin D[4] are two representative members of the *Annonaceae* acetogenins, which show their characteristic structural features: An ether core consisting of tetrahydrofuran (THF) and tetrahydropyran (THP) rings flanked with a left and a right side chain. At the end of the right side chain is located a butenolide unit. The long alkyl chains place at least parts of the compound in the lipophilic interior of the mitochondrial membrane.[5]

It has been proposed that the annonaceous acetogenins act at the terminal electron-transfer step of complex I between the Fe–S cluster N2 and the ubiquinone pool.[2] The butenolide subunit may bind at the quinone binding site of complex I. The reduction potential of an α-alkyl-α,β-unsaturated butyrolactone ($E_p^c = -2.69$ V (irrev.), CH_3CN, versus the saturated calomel electrode (SCE)) is much more negative than the reduction potentials of the quinone group ($E_p^{cI} = -0.75$ V, $E_p^{cII} = -1.48$ V, CH_3CN, versus SCE).[6] Therefore an electron transfer from the Fe–S cluster N2 to the butenolide is unlikely to occur. With the goal to further elucidate the mechanism of action of the acetogenins and to provide molecular probes for complex I studies we designed quinone–mucocin **1** and quinone–squamocin D **2**. In these compounds the butenolide part of mucocin and squamocin D is exchanged against the quinone part of ubiquinone. Here we

[*] Prof. Dr. U. Koert, Dipl.-Chem. S. Hoppen, Dipl.-Chem. U. Emde, Dr. L. Grubert
Institut für Chemie der Humboldt-Universität zu Berlin
Hessische Strasse 1-2, 10115 Berlin (Germany)
Fax: (+49) 30-2093-7266
E-mail: koert@chemie.hu-berlin.de

Dr. T. Friedrich
Institut für Biochemie
Heinrich-Heine Universität Düsseldorf (Germany)

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