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Ultra-selective, ultra-clean 1D rotating-frame Overhauser effect spectroscopy†

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An ultra-selective 1D NMR experiment – GEMSTONE-ROESY – enables clear, unambiguous assignment of ROE signals in the not uncommon situation that traditional selective methods fail. Its usefulness is demonstrated in the analysis of the natural products cyclosporin and lacto-*N*-difucohexaose I, providing detailed insight into the structures and conformations of these molecules.

Solution-state nuclear magnetic resonance (NMR) spectroscopy is a versatile and powerful tool, which provides invaluable data about molecular structure and dynamics of molecules in solution. The structural information gained by NMR is unmatched by other solution-state analytical methods. Classically, 2D NMR methods¹ are often used for structure elucidation of small molecules, providing a full profile of bond connections or through-space interactions of a molecule. However, 2D methods can be very time-consuming. Alternatively, direct selection of key signals can in many cases provide the required information for structural analysis more efficiently. In such instances, 1D selective experiments² are fast alternative approaches, taking a fraction of the time of their 2D counterparts. One of the main barriers to the use of 1D selective methods is their inability to excite a single signal selectively when multiplets overlap. Signals can be resolved and identified using pure shift NMR approaches,^{3–6} but attempts at frequency-selective excitation of a single multiplet from an overlapped region using conventional 1D selective experiments give ambiguous results. Recent developments have led to the

production of an ultra-selective method, GEMSTONE (gradient-enhanced multiplet-selective targeted-observation NMR experiment)⁷ which provides selectivity similar to that of the previously state-of-the-art ultra-selective method, the CSSF (chemical shift selective filter),⁸ but in a single transient. Unlike CSSF, GEMSTONE retains the full time advantage of selective 1D over 2D experiments. GEMSTONE has recently been demonstrated with common structure elucidation methods such as NOESY^{7,9} (nuclear Overhauser effect spectroscopy) and TOCSY^{10,11} (total correlation spectroscopy), providing detailed through-space and through-bond structural information, respectively.

The NOE (nuclear Overhauser effect) is the transfer of magnetisation *via* cross-relaxation between nuclei that share a dipolar interaction. Such through-space interactions give important insights into molecular conformation and configuration, which can greatly aid structure elucidation.¹² NOEs can be either positive or negative depending on the magnetic field strength used and the dynamic properties of the species observed.^{9,13} A clear issue arises when these positive and negative contributions cancel. This is typically seen for molecules with a molecular weight in the region of 1000–2000 g mol^{−1}, depending on the solvent viscosity and the magnetic field strength of the NMR spectrometer. The ROESY (rotating-frame Overhauser effect spectroscopy) experiment was developed to circumvent this issue.¹⁴ While NOEs can be positive or negative (or zero), ROEs are always positive. Here a new 1D GEMSTONE-ROESY method is therefore proposed as an ultra-selective method for observing through-space interactions where NOESY experiments fail to provide useful data.

Cyclosporin, an oral immunosuppressant used to prevent organ transplant rejection, falls into the 1000–2000 g mol^{−1} molecular weight range and generally shows poor NOEs.¹⁵ Unexpectedly, cyclosporin is orally active despite failing to meet the usual requirements for bioavailability;¹⁶ it is a “beyond the rule-of-five” compound. A key reason for its oral activity is the protean nature of cyclosporin, which adopts different conformations in different chemical environments.¹⁷ Analysis of 3D conformation is therefore vital to understanding how it behaves in different contexts, in addition to facilitating structure elucidation. The need

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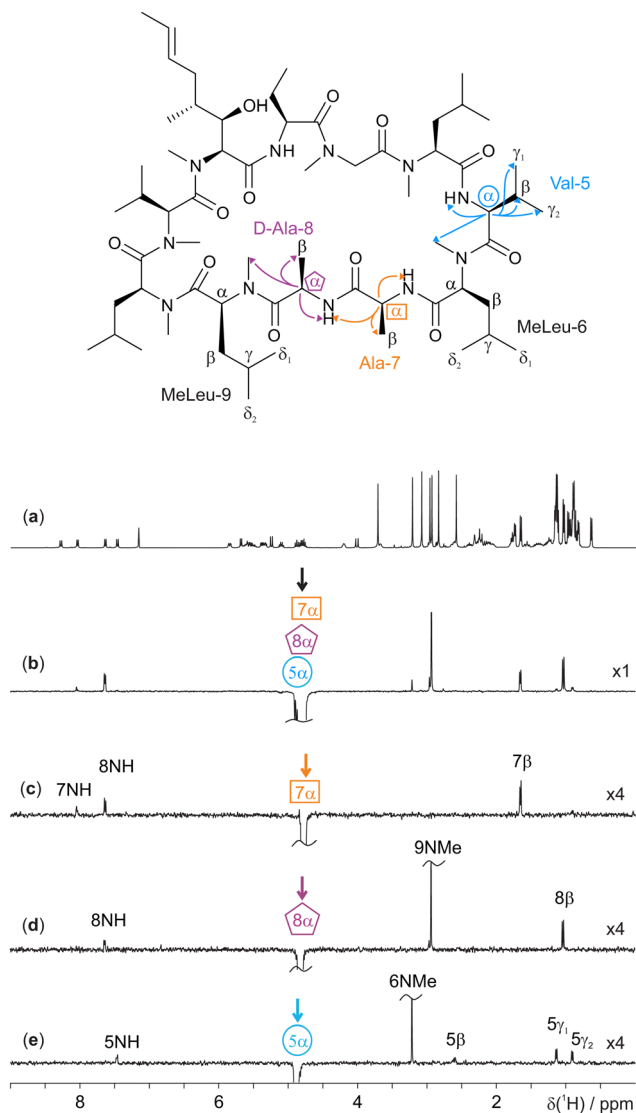


Fig. 1 400 MHz ^1H NMR spectra of 70 mM cyclosporin in C_6D_6 . Molecular structure with relevant assignments¹⁸ is shown above the spectra, with arrows representing the ^1H - ^1H ROEs observed; for simplicity, individual protons are not shown. (a) Conventional ^1H 1D spectrum, (b) 1D selective EASY-ROESY centred at 4.82 ppm using a 20 Hz selective refocusing pulse, and (c–e) GEMSTONE-ROESY spectra exciting at 4.78 (Ala-7 α), 4.82 (D-Ala-8 α), and 4.88 (Val-5 α) ppm, respectively. The GEMSTONE adiabatic pulse duration was set as 100 ms and a selective refocusing pulse with a 200 Hz bandwidth was used. All ROESY spectra were acquired using a 200 ms spin-lock mixing period. Further experimental details are given in the ESI.† Spectra (c–e) are scaled by a factor of 4 to match the intensity of the spectrum in panel b.

for ultra-selective excitation methods such as GEMSTONE-ROESY is apparent in the extensive multiplet overlap present in several regions of the ^1H NMR spectrum of cyclosporin (Fig. 1a). Very weak NOEs are observed (see ESI,† Fig. S3) highlighting the need for ROESY experiments if through-space interactions are to be measured.

GEMSTONE ensures that only on-resonance signals are retained, while all off-resonance signals are dephased.⁷ It therefore requires that the exact chemical shift of the signal

of interest be known. Pure shift methods^{3–6} offer the ability to measure chemical shifts without multiplet structure; a single peak at the chemical shift is observed for each chemical environment. In this case, a PSYCHE (pure shift yielded by chirp excitation) pure shift ^1H NMR spectrum was acquired (see ESI,† Fig. S2).^{19,20} The three overlapping multiplets in the spectral region 4.7–4.9 ppm (Fig. 1a) correspond to the alanine-7 α (4.78 ppm), D-alanine-8 α (4.82 ppm) and valine-5 α (4.88 ppm) signals,¹⁸ with chemical shifts which are seen to be approximately 20 Hz apart in the pure shift spectrum. As expected, a conventional 1D selective EASY-ROESY^{21,22} spectrum (Fig. 1b) struggles to distinguish between the three resonances. All three overlapped signals are excited, to different extents, resulting in ROE peaks that cannot be assigned unequivocally to a given proton. Ilgen *et al.* recently published a pure shift 2D EASY-ROESY method to resolve the observed ROE signals better.²² Here, an alternative, faster solution is presented in the form of ultra-selective 1D excitation. This retains the advantage of short experiment time, and excites one chemical shift at a time, allowing ROEs to be assigned directly. This application of the ultra-selective GEMSTONE-ROESY method is illustrated in Fig. 1c–e, where the previously inseparable signals have been individually selected. In contrast to Fig. 1b, Fig. 1c–e show ROEs originating in magnetisation transfer from a single resonance. Enabling the selection of individual α -protons allows the signals of each amino acid residue to be identified, along with those of adjacent residues.

The new GEMSTONE-ROESY pulse sequence is shown in Fig. 2. The 'EASY-ROESY' mixing element has been used here, to attenuate TOCSY interference, along with zero-quantum coherence (ZQC) suppression elements.²³ The ultra-selective nature of the experiment originates from the GEMSTONE element.⁷ The combination of dual swept-frequency pulses with simultaneous pulsed field gradients (PFGs), labelled G_1 , causes all signals within the sample to become spatially encoded. Only on-resonance signals survive, all off-resonance signals are dephased. The selectivity of the experiment is inversely proportional to the total duration of the swept-frequency pulses. However, sensitivity decreases with increased duration, due in part to relaxation, so a compromise must be made between selectivity and sensitivity. The GEMSTONE-ROESY sequence uses additional PFGs to minimise subtraction artefacts,

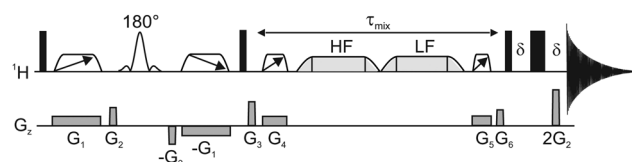
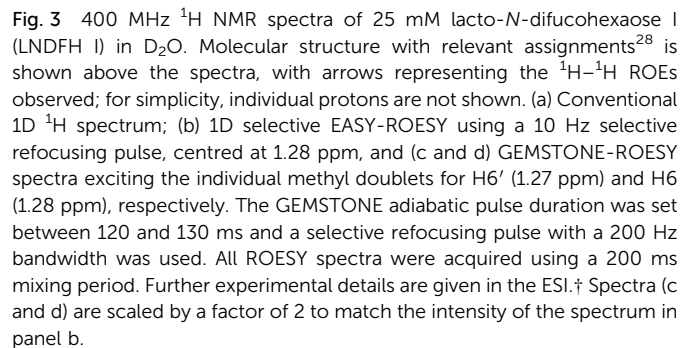


Fig. 2 Schematic representation of the 1D GEMSTONE-ROESY pulse sequence. Narrow and wide rectangles represent hard 90° and 180° radiofrequency pulses, respectively. The open trapezoids with directional arrows are adiabatic 180° pulses used for GEMSTONE selection and ZQC suppression. The labelled 180° pulse shape denotes a band-selective refocusing pulse. The spin-lock element is shown by light grey trapezoids, held first at a high-field (HF) and then at a low-field (LF) offset. The mixing period, τ_{mix} , incorporates the spin-lock and the ZQC suppression elements. Pulsed field gradients are represented by grey boxes in the G_z channel. The delay δ is just sufficient for the gradient pulse and recovery delay. Further details of the pulse sequence are given in the ESI.†

The new ultra-selective, ultra-clean 1D GEMSTONE-ROESY experiment has been shown to provide unambiguous ROE data even in spectral regions of severe multiplet overlap. GEMSTONE-ROESY offers a simple alternative where traditional 1D selective



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Conflicts of interest

There are no conflicts to declare.

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