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A Method to Observe NOE from Regions of Spectral Overlap. Nuclear-Overhauser-Enhanced *J*-Resolved Difference Spectroscopy

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The nuclear Overhauser effect is an important tool in the identification of compounds by NMR. The ''effect'' is a result of dipolar relaxation between two or more spins within 3 Å of each other. This internuclear distance information is used to determine (1) the relative stereochemistry of a molecule, (2) the conformation of a molecule, and/or (3) the assignment of individual resonances. There are two principal methods to observe nuclear Overhauser enhancements, and they are the two-dimensional NOESY- and ROESY-type experiments and the 1D NOE difference experiment.

The intensity of the nuclear Overhauser enhancement is dependent upon a number of factors. For small molecules, the important considerations are the correlation time for molecular tumbling, the distance between mutually relaxing nuclei, the number of interacting nuclei, and the degree of saturation of the target nucleus (or the mixing time in transient experiments). These items all conspire to make the 1D NOE difference experiment the technique of choice for small-molecule identification.

The problem with any 1D experiment is extracting information from regions of spectral overlap. It is quite common to observe an NOE originating from a region of overlapping multiplets and be unable to assign it to a particular proton. This Note describes an experiment, *n*uclear-*O*verhauser-*e*nhanced *J*-*re*solved *d*ifference spectroscopy (NOE J-REDS) that can be used to alleviate this problem. It is premised upon the fact that a 2D J-resolved spectrum resolves overlapping signals by displaying multiplets orthogonal (after tilting 45°) to the chemical-shift axis (*1*). In this manner, the signals are observed as a series of columns and can be readily differentiated from their overlapped partners. The NOE J-REDS pulse program produces a *J*-resolved 2D spectrum that consists solely of signals (columns) manifesting NOEs to an irradiated proton and, by reference to a *J*-resolved 2D experiment, readily pinpoints the nuclear-Overhauser-enhanced signal from amongst the overlapping multiplet. This experiment is particularly suited for determining NOEs in small highly saturated molecules.

The experiment consists of a standard 2D *J*-resolved spin-echo sequence preceeded by a low-power CW irradiation of the target nucleus (Fig. 1a). The subsequent experiment moves the frequency of CW irradiation off resonance and changes the receiver phase 180° to effect subtraction. Artifact suppression is accomplished by phase cycling this pair of experiments using the EXORCYCLE (*2*) phase-cycling scheme. This may then be followed by 90° incrementation of the pulse and receiver phases to yield, ultimately, a 32-step cycle. The net result is the 2D *J*-resolved equivalent of a 1D NOE difference experiment.

The strategy for applying the technique begins with the assignment of all, or most, resonances via H–H, C–H, and long-range C–H (H–C–C and H–H–C) scalar interactions via the appropriate 2D NMR experiments (*3, 4*). The C–H correlation experiment should be obtained in sufficient digital resolution in the proton dimension to determine the order of signals in spectrally overlapped areas. A *J*-resolved experiment should then be performed, and the overlapped regions differentiated according to the order derived from the C–H correlation experiment. Finally, NOE J-REDS is performed, irradiating the nucleus of interest. Signals manifesting an NOE, in the NOE J-REDS experiment, can then be assigned by reference to the *J*-resolved spectrum.

The utility of this experiment is illustrated by the analysis of megestrol acetate, Fig. 1b, a drug effective as an antineoplastic for breast cancer and as a palliative treatment for weight loss in AIDS patients. In this instance, the NOE J-REDS experiment deconvoluted NOEs observed in two overlapped regions of the ^{1}H NMR spectrum and, in one of them, revealed the presence of an NOE that was not observed in the 1D difference experiment.

The most congested area of the ^{1}H NMR spectrum of megestrol acetate, Fig. 2, was the region between δ 2.05 and 1.35. The *J*-resolved spectrum of this region is presented in Fig. 3, and the resonances were assigned with NMR experiments as described above. Within the region δ 2.05 to 1.35, two strongly overlapped regions were between 2.04 and 1.97 (H1 and H12) and 1.52 and 1.36 (H15 and H11). These two areas were deciphered by observation of the NOEs asso-

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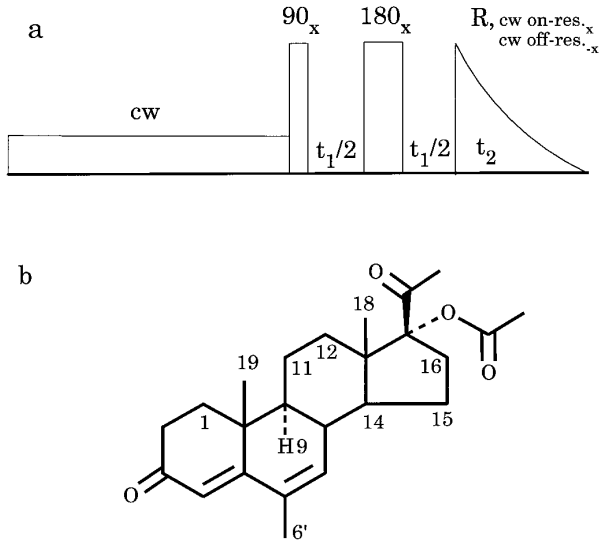

FIG. 1. (a) The NOE J-REDS pulse sequence. A 32-step phase cycle was used to suppress artifacts (see text). (b) The structure and numbering scheme of megestrol acetate.

ciated with the axial methyl groups of this steroid: The $CH_3$18 irradiated experiment, Fig. 4a, revealed an enhancement in the region δ 1.52–1.36, but it was difficult to determine if one or both resonances were involved. An NOE J-REDS (Fig. 4b), however, deconvoluted the signals and revealed an NOE to both resonances, thus assigning them as H15β and H11β. In addition, signals were observed at an F_2 frequency of 1.58 and 1.99. Since the axial methyls are probes of the β plane of the molecule, these signals were identified as H12β and H12α, respectively. Note that the NOE to H12α was not observed in the 1D experiment, Fig. 4a.

The observation of an NOE to H12α was not anticipated based on the results of the 1D NOE difference experiments. Incongruent results between the two techniques, such as this, usually occur for small NOEs and may simply be due to a poor signal/noise ratio. Alternatively, it may be due to the fact that the signals in a 1D NMR spectrum are a continuum and overlapped signals, such as from subtraction artifacts or positive and negative NOEs, can add in such a way as to give a false result. NOE J-REDS removes these interferences by deconvoluting the overlapped resonances. This provides for a much more sensitive experiment in the sense that smaller NOEs may be observed in the presence of artifacts. These may be indirect NOEs or NOEs from hydrogens more distant in space. For example, in the case of the irradiation of $CH_3$18, the enhancement to H12α in the region δ 2.04–1.97 was an indirect NOE.

A further analysis of the region δ 2.04–1.97 was undertaken by irradiation of $CH_3$19. A weak NOE was observed in the 1D NOE difference experiment, Fig. 5a, and as above, due to spectral overlap it was not possible to determine if one or both resonances were involved. The NOE J-REDS,

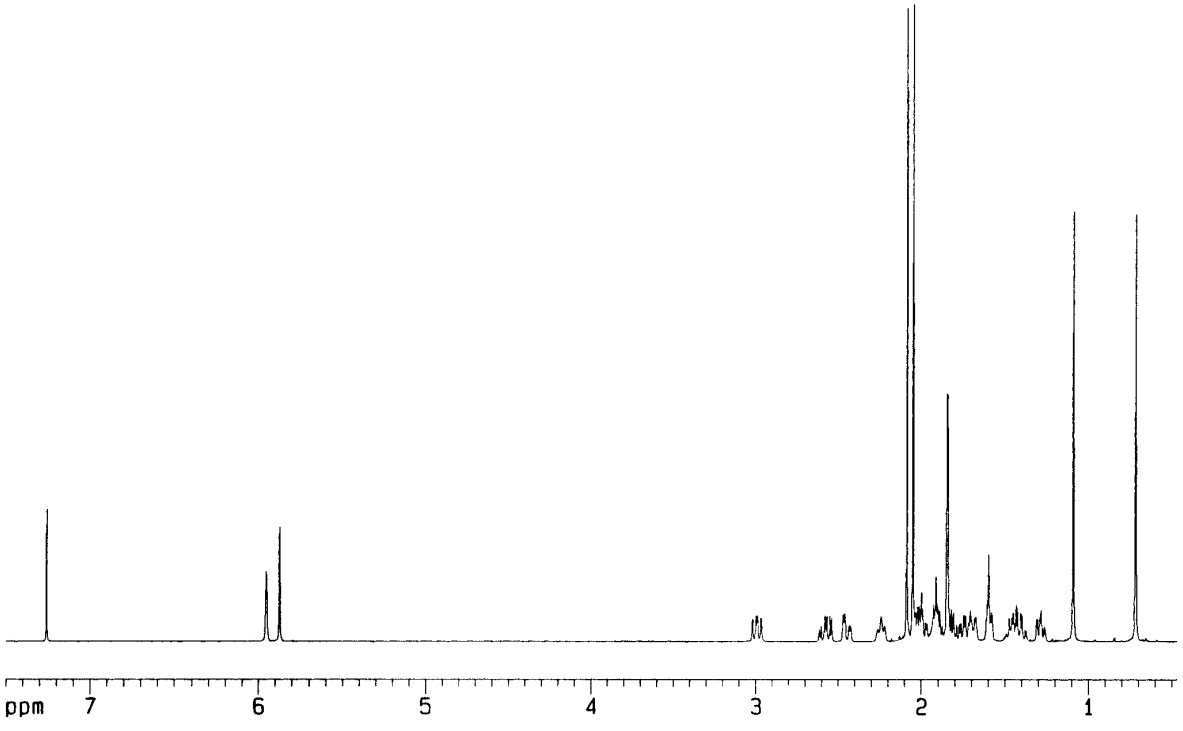

FIG. 2. The ^{1}H NMR spectrum of megestrol acetate.

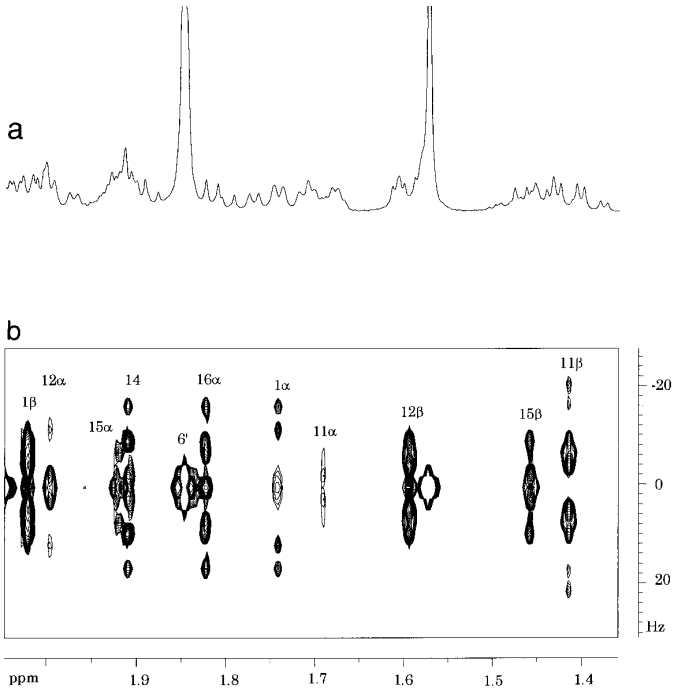

FIG. 3. (a) An expansion of the ^{1}H NMR spectrum between δ 2.05 and 1.35. (b) The *J*-resolved spectrum of this region. For convenience, the stereochemistry of the signals were labeled in this figure but were actually assigned subsequently, based on the NOE J-REDS described in the text.

Fig. 5b, revealed an enhancement to just one of the signals in this region, and it was identified as H1β. A second NOE was observed in Figs. 5a and 5b, and it coincided with one of the two signals in the region δ 1.52–1.36, H11β.

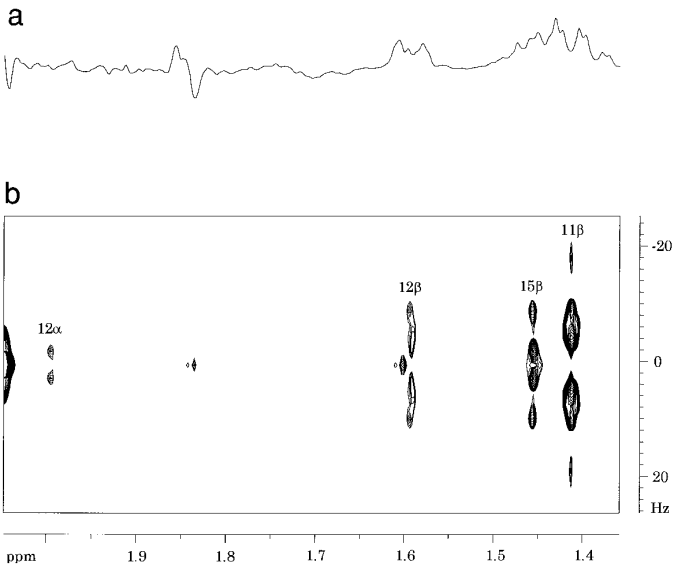

FIG. 4. NOE experiments irradiating $CH_3$18, δ 0.69: (a) The 1D NOE difference experiment and (b) the NOE J-REDS.

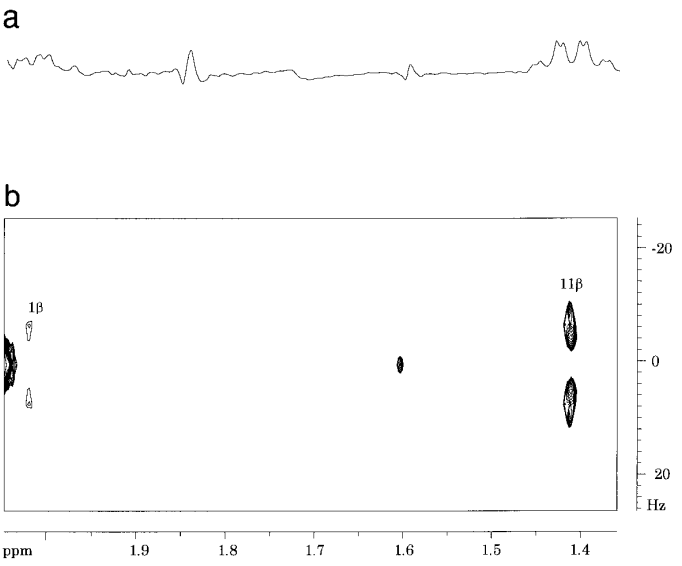

FIG. 5. NOE experiments irradiating $CH_3$19, δ 1.07: (a) The 1D NOE difference experiment and (b) the NOE J-REDS.

The above analyses described two situations in which use of NOE J-REDS would be beneficial: The resolution of observed NOEs in overlapped regions of the spectrum to determine if one or more signals were involved, and the inspection of regions of overlap that appear absent of NOE to ensure against false results. Contrary to the results of the 1D difference experiment, regions that appear devoid of NOE (Figs. 4a and 4b, δ 2.04–1.97) often reveal important structural information.

The observation of a signal in an NOE J-REDS experiment may be due to a positive NOE, a negative NOE, a selective population transfer (SPT) (*5*), or partial saturation of a resonance adjacent to the irradiation site. In the 1D difference experiment, it is possible to distinguish these events based on lineshape. In the NOE J-REDS, however, this is not possible because the experiment is processed as a power spectrum. Accordingly, a negative NOE, which would indicate an indirect transfer of dipolar relaxation, can only be inferred based on prior knowledge of the structure. Also, if the target nucleus is a multiplet, and the irradiation power does not saturate all components to an equal extent, selective population transfer can occur. This event would not be differentiated from an NOE in the NOE J-REDS. Finally, if the resonance of interest is near the target nucleus, partial saturation may occur. This, again, could be misinterpreted as an NOE in the NOE J-REDS experiment. For these reasons, the 1D NOE difference experiment should be performed in conjunction with the 2D experiment.

The appearance of artifacts at $F_1 = 0$ in NOE J-REDS is coincident with the presence of singlets in the ^{1}H NMR spectrum. For example, in Fig. 4b, the signals at $F_2 = 2.05$ and 1.83 were from methyl groups and the signal at δ 1.60

was from residual water in the sample. The observation of NOEs to methyl groups are, therefore, best determined with the 1D experiment.

These experiments were performed on a Bruker AMX spectrometer operating at 500.13 MHz for the observation of protons. The NOE J-REDS experiments were performed on a 6 mg sample of megestrol acetate dissolved in 0.4 ml $CDCl_3$. Spectral data were referenced to residual $CHCl_3$ at $\delta = 7.26$. CW irradiation for both the on- and off-resonance part of the pulse sequence lasted 3 s at a power of 0.18 μW. The frequency of the off-resonance cycle was shifted approximately 10,000 Hz from the on-resonance cycle. Sixteen dummy scans were acquired at the start of the experiment, and 32 transients were acquired at each t_1 increment. The spectral width was 4800 Hz and 300 Hz in t_2 and t_1, respectively. Two kiloword complex points were obtained for each of 128 t_1 increments and zero filled to a 4K $\times$ 128 data table. The data were multiplied with $\pi/32$ (t_2) and $\pi/16$ (t_1) shifted sinebell-squared apodization factors and Fourier transformed into a power spectrum. The spectrum was then tilted 45° to align the multiplets orthogonal to F_2 and then symmetrized about $F_1 = 0$.

The *J*-resolved experiment was performed similar to the above with the exception of the use of 4 dummy scans and 16 transients instead of 16 and 32, respectively. The sample of megestrol acetate and the amount used, 3 mg, were also different. Thus, the cross peak for residual water is more intense and at a different shift, 1.57, in Fig. 3 than in Figs. 4 and 5. The NOE difference experiments were performed by acquiring 16 dummy scans and 16 transients for both the on- and off-resonance experiments. The total irradiation time, as above, was 3 s at a power of 0.18 μW. These two experiments were then cycled 4 times, for a total of 64 acquisitions each. The data were processed with 2 Hz exponential multiplication and the two spectra subtracted after Fourier transformation.

In conclusion, the NOE J-REDS experiment is a complementary technique to the 1D NOE difference experiment when regions of spectral overlap need to be investigated. The problems observed with the 1D difference experiment are often associated with weak enhancements and are normally due to overlap of the signal with subtraction artifacts and/or multiplets experiencing positive or negative NOE. The NOE J-REDS experiment deconvolutes these effects by displaying a *J*-resolved spectrum which consists only of those protons manifesting an NOE to a selected nucleus. In addition, the better resolution of the 2D NOE J-REDS experiment can effectively increase the sensitivity of the NOE measurement by isolating spins from subtraction artifacts.

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