Communications to the Editor

Diffusion-Edited NMR−Affinity NMR for Direct Observation of Molecular Interactions

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Combinatorial chemistry enables the preparation of large numbers of compounds for testing in drug discovery programs in the pharmaceutical industry.[1,2] The synthesis and screening of mixtures of compounds offers increased efficiency and throughput compared to making and testing individual compounds. However, utilization of mixtures of compounds requires a method to determine which molecule in the mixture is responsible for the desired effect. Typically, mixtures of compounds are prepared by design and these mixtures are tested without separation. When there is evidence of sufficient activity, the mixture is deconvoluted to identify the active component. Several approaches[3] to identify interesting components in a mixture have been described, including iterative rescreening of subsets of the mixture, recursive deconvolution, synthesis of the mixture in pools allowing direct deduction of the interesting structure, and tagging. In the worst-case scenario for testing mixtures, no individual components can be identified, and the observed "false positive" response is a result of an additive effect of all the compounds in the mixture. Methods which can identify active components of mixtures without the need for deconvolution could eliminate such "false positives" and greatly reduce the effort required to analyze mixtures. One such method under investigation is affinity mass spectroscopy.[4]

We have been interested in developing NMR methodologies to aid mixture analysis without the need of prior separation of the components and have developed a technique called diffusion encoded spectroscopy (DECODES), which involves pulse field gradient (PFG) NMR combined with total correlation spectroscopy (TOCSY).[5] We have demonstrated that the structure of molecules in small mixtures can be determined without prior separation of the components. This methodology relies on PFG NMR which spatially encodes molecules in solution and enables structure determination of the individual components due to differences in diffusion coefficients.[6]

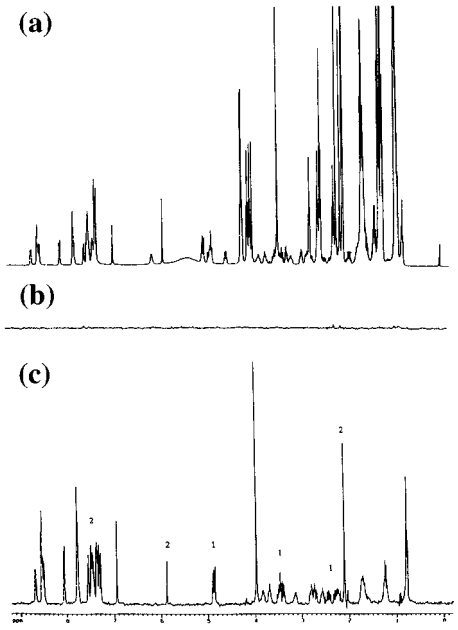
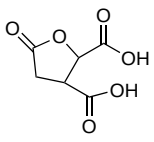


Figure 1. (a) 1D 400 MHz ^{1}H NMR spectrum of the nine-component mixture in $CDCl_3$. The concentration of each component is 10 mM. Components: (**1**) DL-isocitric lactone, (**2**) (*S*)-(+)-*O*-acetylmandelic acid, (**3**) DL-*N*-acetylhomocysteine thiolactone, (**4**) (±)-*sec*-butyl acetate, (**5**) propyl acetate, (**6**) isopropyl butyrate, (**7**) ethyl butyrylacetate, (**8**) butyl levulinate, (**9**) hydroquinine 9-phenanthryl ether. (b) 1D PFG ^{1}H NMR spectrum of the mixture without hydroquinine 9-phenanthryl ether using LED sequence.[9] (c) 1D PFG ^{1}H NMR spectrum of the nine-component mixture. Chemical shifts arising from compounds **1** and **2** are labeled. All other shifts are from compound **9**. The PFG conditions were the same as in the middle spectrum.

PFG conditions can be established by which all the resonances of small molecules in the molecular weight range of 200−400, typical of many organic compounds, will disappear from the NMR spectrum. We reasoned that if the apparent size of a small molecule could be altered by complexation with a partner in solution, the diffusion coefficient of the complex should be significantly different from the compounds that do not interact, in a manner reminiscent of separation by affinity chromatography. This difference in diffusion coefficient should permit the reappearance of the resonances of the small molecule.

To test this notion, we first performed three PFG experiments: (1) with quinine alone, (2) with compound **1** alone, and (3) with a 1:1 mixture of quinine and **1**. Quinine and compound **1** were selected for this model study since we knew from previous work that they formed a complex providing a method whereby we could readily distinguish enantiomeric purity.[7]



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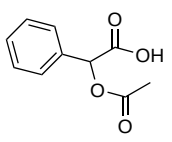

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The experimental PFG conditions have been selected such that no NMR signals could be observed from either compound

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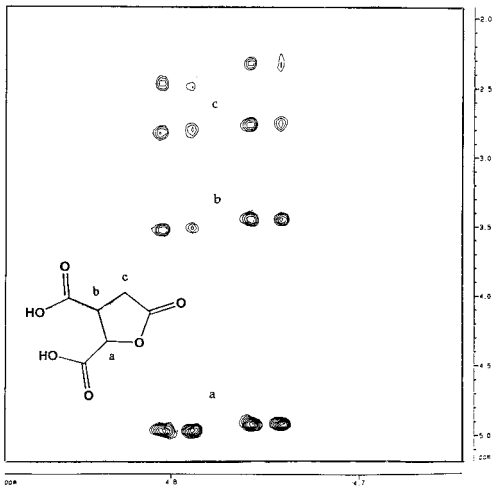

Figure 2. DECODES spectrum of compound **1**. PFG conditions were the same as described in Figure 1.

when they were studied alone.[8] When the two compounds were mixed and a 1D NMR spectrum collected under the same conditions where previously no signal could be observed, the NMR spectrum of each of the components was detected.

Encouraged by this positive result, we replaced quinine with hydroquinine 9-phenanthryl ether (**9**) and expanded our study to a mixture of nine compounds containing the two components (**1** and **2**) known to interact with quinine. Since we are dealing with an equilibrium situation of free and bound substrates, which is fast on the NMR time scale, to a first approximation, the larger the transient complex, the greater the sensitivity of the experiment. The replacement of quinine with **9** would be expected to enhance the differentiation of the diffusion constants between the complex and those molecules which do not interact since a larger complex would be obtained.

The results of this set of experiments are shown in Figures 1 and 2. Figure 1 shows the normal 1D ^{1}H NMR spectrum for the nine-component mixture without PFG (a), and the 1D ^{1}H NMR spectrum of the same mixture under the PFG conditions is depicted (c). The only signals that are observed are the signals from hydoquinine 9-phenanthryl ether and compounds **1** and **2**. A control experiment performed on the mixture in the absence of hydroquinine 9-phenanthryl ether under identical PFG conditions is also shown in Figure 1b. No NMR signals are present, as expected, in the absence of molecular interactions.

The structures of the compounds that interact with hydroquinine 9-phenanthryl ether can be identified directly in the mixture without resorting to physical separation by consideration of their chemical shifts or by using our DECODES method. Figure 2 shows the 2D DECODES spectrum of compound **1**, using the same PFG conditions as in the 1D experiment. Evaluation of the TOCSY data allows not only the identification of compound **1** but also the observation of the two enantiomer components.

In conclusion, we have shown that by using PFG techniques, a small mixture of compounds can be selectively edited to find and identify components involved in molecular interactions. The structure of the interacting ligand can be deduced directly, without separation of the mixture, using the DECODES pulse sequence. The practical application of this new methodology to screen combinatorial chemistry mixtures for biological activity will likely be limited by the total compound concentration tolerated by the biological target, since the relatively high concentration of each component required by NMR quickly adds up to a high total concentration of compounds for the mixture.[10] Nevertheless, this NMR method should add a powerful tool for mixture analysis when applied to suitable biological systems and for other systems of molecular recognition.

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(8) The NMR spectra were obtained on a Bruker DMX-500 spectrometer with gradient accessories. For the gradient-edited experiments, the gradient pulse strength was 0.394 T m^{-1}, the gradient pulse duration time was 2 ms and the diffusion delay time was 0.15 s. The DECODES spectrum was acquired using the same PFG conditions as in the 1D spectra with a spin-lock time of 70 ms. The spectrum was obtained using a sweep width of 6024 Hz. A total of 256 complex increments were collected in t_1, and 2K data points were collected in t_2. The data was processed using a unshifted sine bell apodization in both dimensions.

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(10) Other potential limiting factors could be excessive signal broadening depending on the rate of ligand exchange between free and bound forms, or short transverse relaxation times (T_2s) of the bound ligand resulting in diminished signal intensities.