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Kinetic and *J*-Resolved Statistical Total Correlation NMR Spectroscopy Approaches to Structural Information Recovery in Complex Reacting Mixtures: Application to Acyl Glucuronide Intramolecular Transacylation Reactions

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We demonstrate here a new variant on a statistical spectroscopic method for recovering structural information on unstable intermediates formed in reaction mixtures. We exemplify this approach with respect to the internal acyl migration reactions of 1-β-*O*-acyl glucuronides (AGs), which rearrange at neutral or slightly alkaline pH on a minute to hour time scale to yield a series of positional glucuronide ring isomers and α/β anomers from the 1-β (starting material), i.e. 2-β, 2-α, 1-α, 3-β, 3-α, and 4-β, 4-α isomers together with the aglycon and α- and β-glucuronic acid hydrolysis products. Multiple sequential 800 MHz cryoprobe ^{1}H NMR spectra (1D and 2D *J*-resolved, JRES) were collected on a 5.1 mM solution of a synthetic model drug glucuronide, 1-β-*O*-acyl (*S*)-α-methyl phenylacetyl glucuronide (MPG) in 0.1 M sodium phosphate buffer in D_2O at pD 7.4 over 18 h to monitor the reaction which leads to the formation of the eight positional isomers and hydrolysis products. As the reaction proceeds and new isomers form, the NMR signal intensities vary accordingly allowing the application of a novel kinetic variant on statistical total correlation spectroscopy (K-STOCSY) method to recover the connectivities between proton signals on the same reacting molecule based on their intensity covariance through time. We performed K-STOCSY analysis on both the standard 1D NMR spectra and the skyline projected singlets of the ^{1}H−^{1}H JRES NMR spectra through time, i.e. the K-JRES-STOCSY experimental variant, which increases the effective spectral dispersion and is ideally suited for the analysis of heavily overlapped spin systems. High statistical correlations were observed between mutarotated α- and β-anomers of individual positional isomers, as well as directly acyl migrated products and anticorrelation observed between signals from compounds that were being depleted as others increased, e.g. between the 1-β and 2-α/2-β isomers. This statistical kinetic approach enabled the recovery of structural connectivity information on all isomers allowing unequivocal resonance assignment, and this approach to spectroscopic information recovery has wider potential uses in the study of reactions that occur on the second-to-minute time scale in conditions where multiple sequential NMR spectra can be collected. JRES-STOCSY is also of potential use as a method for recovering spectroscopic information in highly overlapped NMR signals and spin systems in other types of complex mixture analysis.

Two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopic methods are invaluable molecular structural assignment tools, and can readily be used to identify nuclei from within the same scalar *J*-coupled spin-system or from nuclei close in space *via* dipolar interactions. For less sensitive nuclei or for dilute samples, long total acquisition times can be required for some 2D experiments, for example a ^{1}H−^{1}H 2D total correlation spectrum (TOCSY) data set can take several hours to acquire (at high resolution in F1) on biological samples. In dynamic reactions, real-time NMR observation of the degradation process and structural identification of the products can be nontrivial as the changing speciation results in low sensitivity. One such example of a spontaneous reacting metabolic system is provided by the ester glucuronides of drugs.

Many drugs are metabolized to acyl glucuronides (AGs), and these have been studied extensively, because of their possible reactivity toward proteins and consequent immunotoxicity.[1–4] The 1-β-*O* AG is formed initially as a result of conjugation *via* UDP-glucuronosyltransferase that transfers a glucuronic acid moiety

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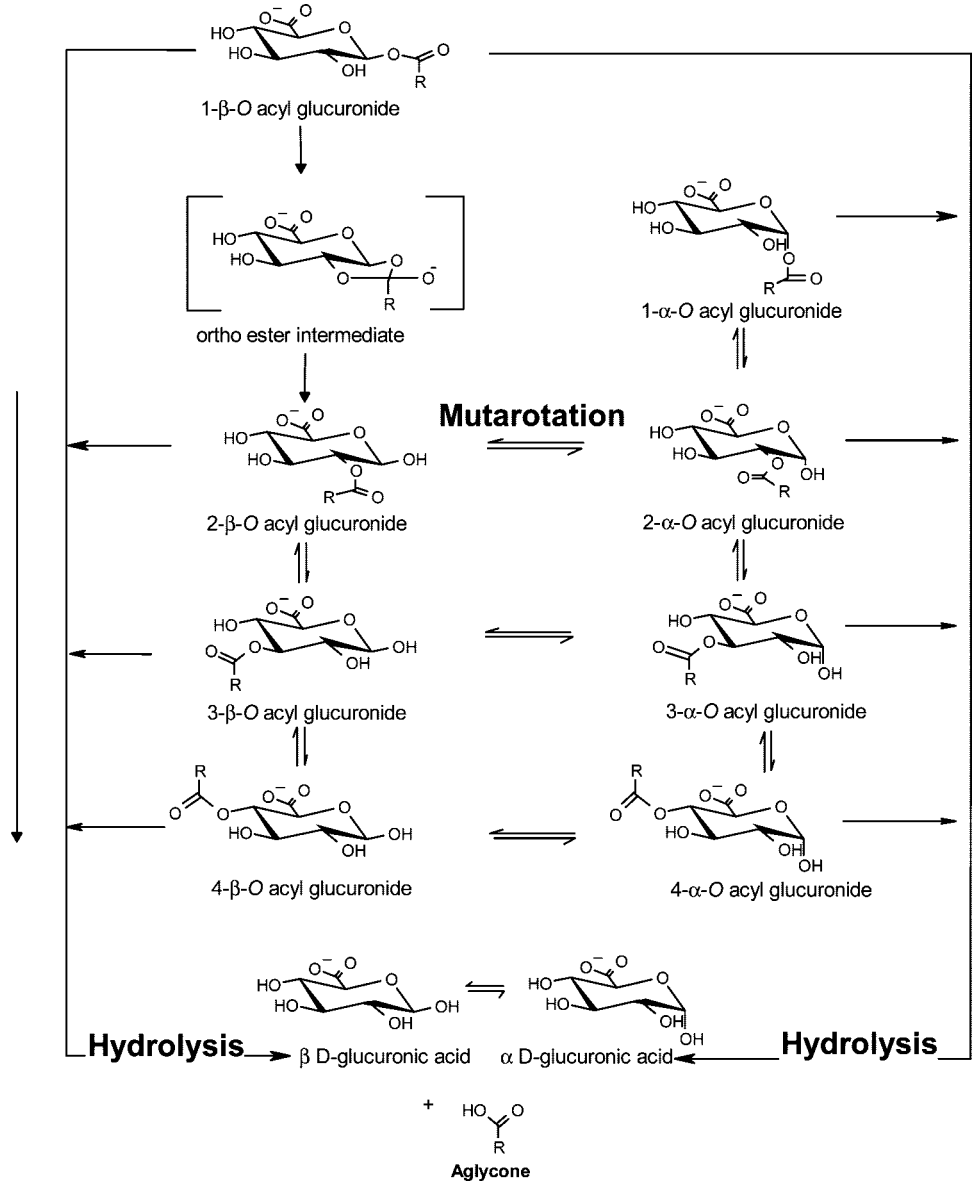
Scheme 1. Rearrangement Reactions of Acyl Glucuronides[a]



[a] All isomers can hydrolyze, but the 1β isomer has the highest rate and contributes most to the aglycon pool.

to a carboxylate group of the drug. This type of metabolic phase II conjugation is common in nonsteroidal anti-inflammatory drugs (NSAIDs) where many commonly used drugs contain a carboxylate group.[5] The resulting 1-β-*O* AG can then undergo a cascade of spontaneous chemical reactions in aqueous solution, resulting in acyl migration (through *ortho*-ester intermediate structures), mutarotation (*via* ring-opened aldehyde forms) and hydrolysis, to produce seven further positional isomers: 2-β, 2-α, 1-α, 3-β, 3-α, 4-β, 4-α the aglycon and α- and β-glucuronic acid as shown in Scheme 1. The rates at which these reactions proceed are a function of the aglycon structure[6,7] (steric and electronic features), the pH[8] and ionic strength of the solution.[1] The positional isomers can also bind irreversibly to endogenous proteins, and the formation of protein adducts by acyl migration can potentially result in immunopathological reactions.[1,3] Several NSAIDs (tolmetin, zomepirac, ibufenac) have been withdrawn from the market, due to different types of adverse reactions.[9,10] Since these withdrawn drugs all have fast AG degradation rates (0.26 h^{-1}, 0.45 h^{-1}, 1.1 h^{-1})[8,11,12] for the 1-β-*O*-AG in aqueous solutions at pH 7.4, and also exhibit protein binding, tentative links have been made between AG toxicity and the AG degradation rate. However, no clear connection has yet been made between simple AG degradation rates and the incidence of reported toxicity,[13] and it is likely that a complex interplay between AG transacylation rates and pharmacokinetic processes is involved.

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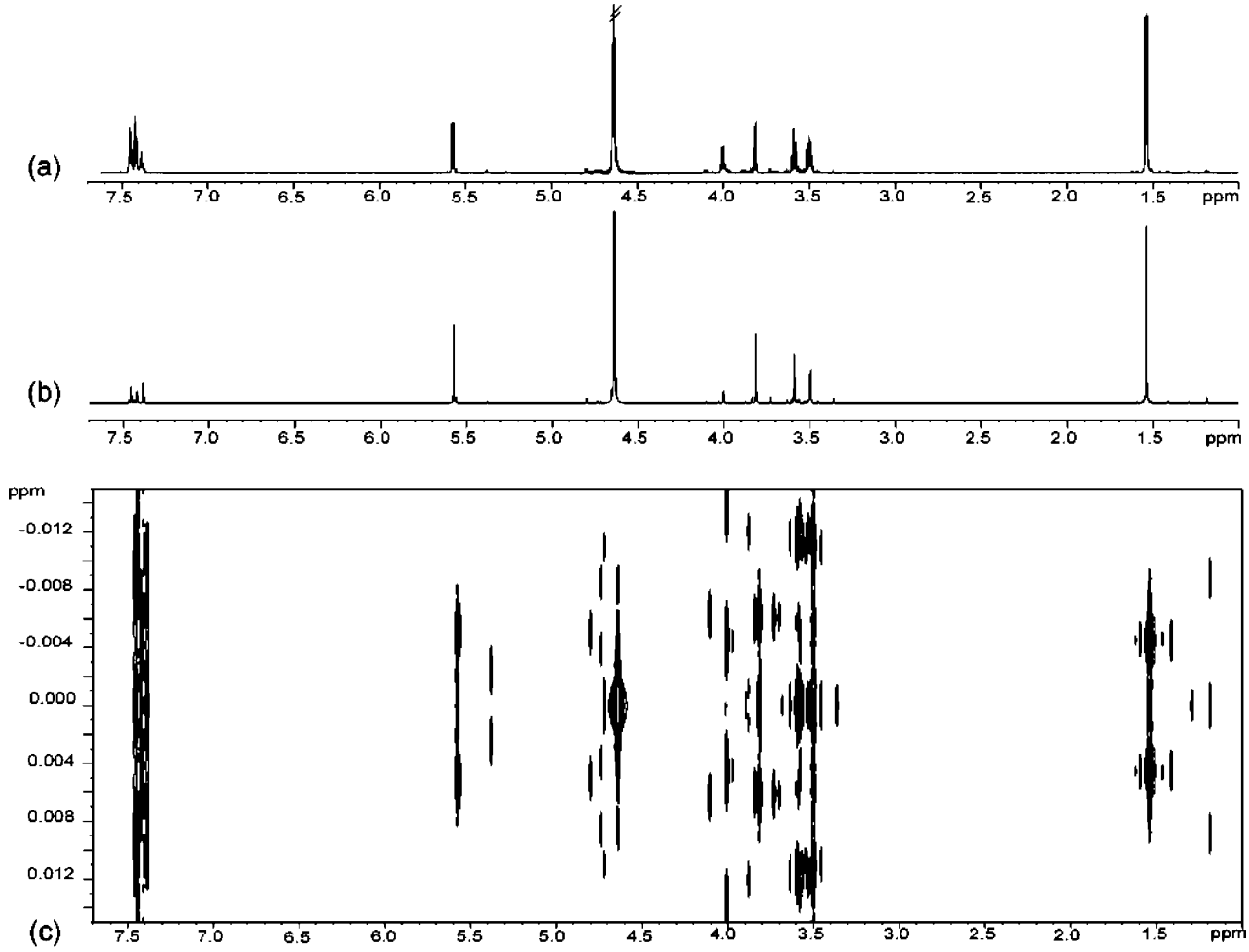

Figure 1. NMR spectra for the (*S*)-α-methyl phenylacetic AG. (a) ^{1}H NMR spectrum. (b) A ^{1}H JRES skyline projection on to the F2 axis. (c) A ^{1}H−^{1}H 2D JRES NMR spectrum.

^{1}H NMR spectroscopy has been applied successfully to study the degradation of 1-*β*-*O* AGs [1,14–26] but resolution and identification of the isomers can be difficult in partly equilibrated or equilibrium mixtures. With the exception of the anomeric proton signals, the glucuronide ring protons are highly coupled and heavily overlapped even at high observation frequencies,[27] such that it is not possible to assign many of the peaks to the positional isomers in partially equilibrated samples using one-dimensional (1D) NMR methods. Kinetic studies have also been performed using ^{19}F NMR spectroscopy for fluorinated drugs,[6] but this gives little direct structural information, without some form of correlated ^{1}H NMR data. ^{13}C NMR spectroscopy has also been applied to study glucuronide transacylation kinetics, but at only 1% ^{13}C natural abundance there are insensitivity problems leading to long spectral acquisition times (usually many hours) even in 1D experiments and consequently few time-points can be analyzed.[28,29] An alternative approach has been to apply liquid chromatography (LC)−NMR to chromatographically resolve individual isomers, which can then be both structurally assigned and held in the NMR probe for individual kinetic experiments;[24,26,30] however, full chromatographic resolution is not always possible with such structurally similar compounds depending on the aglycon structure.

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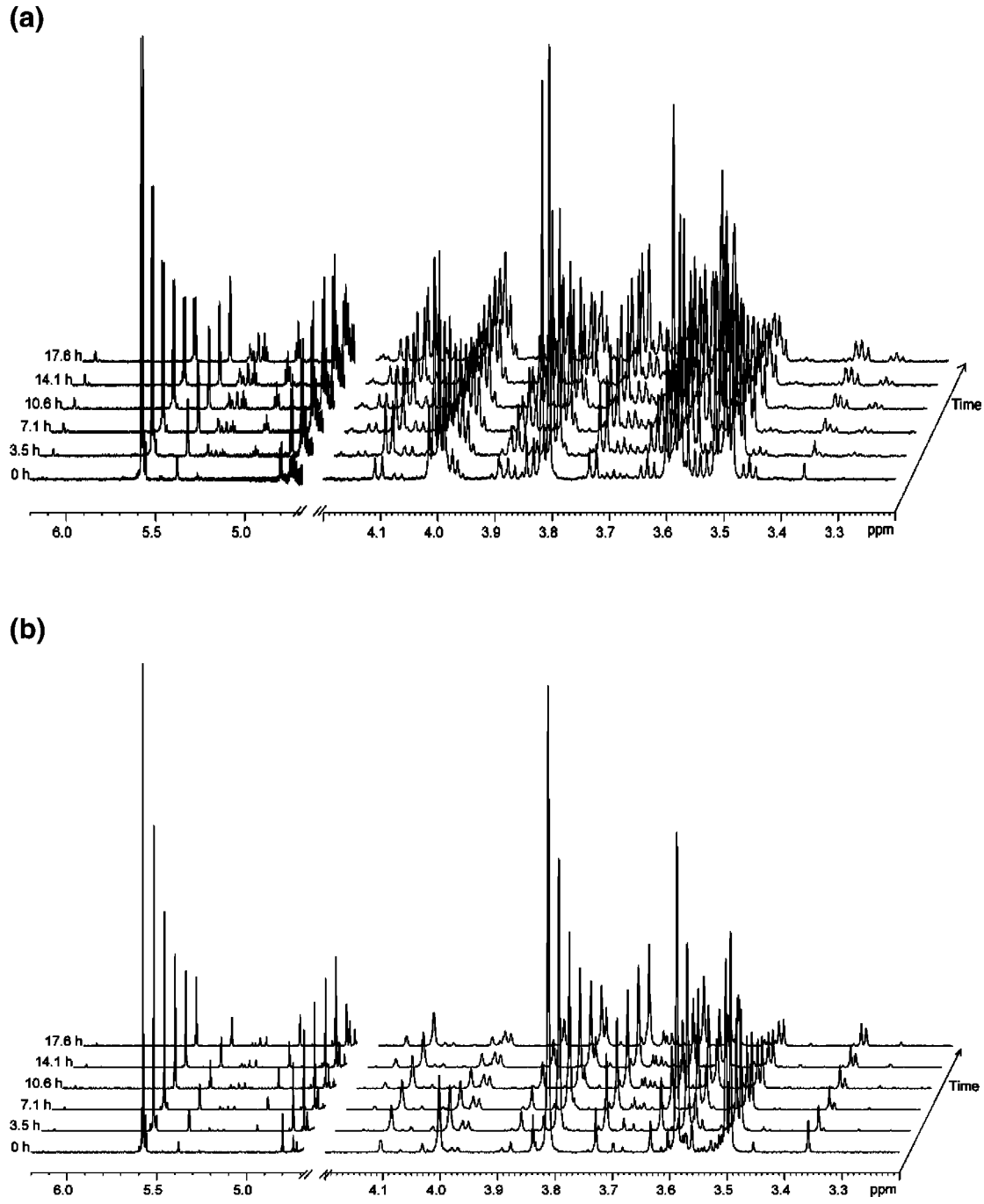

Figure 2. (a) A series of 1D ^{1}H spectra at specific time-points for the glucuronide ring proton resonances. (b) A series of 2D ^{1}H J-RES skyline projections at the same specific time-points for the glucuronide ring proton resonances.

Developments in cryoprobe NMR spectroscopy have permitted much greater real-time sensitivity for most NMR experiments,[31] and the use of higher magnetic fields gives greater frequency dispersion to aid structure elucidation in complex mixtures.[32] The 2D JRES NMR experiment is one of the few 2D approaches that can be performed rapidly (~10 min) with high sensitivity as it requires only a relatively small number of F1 increments to enable effective dispersion of the *J*-couplings and patterns.[33] Tilting and symmetrizing the JRES spectrum after the second Fourier transform allows projection of the spectrum on to the F2 axis giving what is effectively a proton-decoupled proton spectrum (singlets only), which is ideal for studying heavily overlapped spin-systems and gives resolution enhancement through 2D signal processing (use of sine-bell apodization function) and also virtual dispersion improvement.

We have shown previously the value of the JRES approach for studies on small molecule studies in complex biofluids, the ^{1}H NMR spectra of which contain multiple overlapped signals from many metabolites.[1,34–38] However, we have been seeking a more

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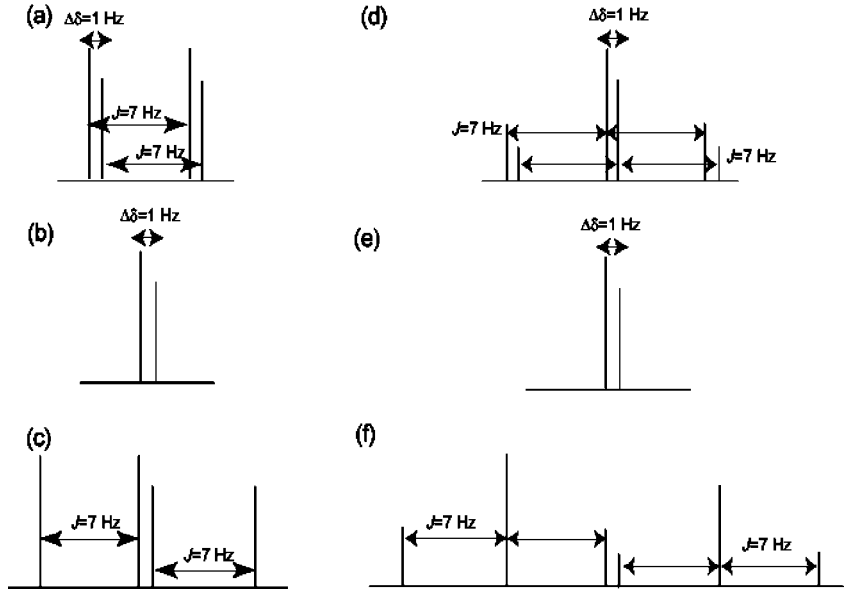

Figure 3. A scheme showing (a) two overlapped doublets, each with $J = 7$ Hz, separated by a chemical shift of 1 Hz; (b) the JRES projection of the two doublets; (c) two doublets showing the inner peaks resolved by 1 Hz, i.e. a chemical shift difference of 8 Hz, requiring an 8-fold increase in NMR observation frequency; (d) two triplets each with $J = 7$ Hz, separated by a chemical shift of 1 Hz; (e) the JRES projection of the two triplets; and (f) the two triplets showing the inner peaks resolved by 1 Hz, i.e. a chemical shift of 15 Hz, requiring a 15-fold increase in NMR observation frequency.

general solution to the problem of overlapped signals in reaction mixtures and have turned to statistical spectroscopic recovery methods, including STOCSY, for which we have found multiple applications in other areas.[39–45] The STOCSY approach to information recovery takes advantage of the colinearity of the intensity variables in a set of NMR spectra, so that correlations from resonances in the same molecule can be identified. The method calculates statistical correlation matrices for individual spectral data points corresponding to peaks of interest, and plots the correlation coefficients onto a pseudo NMR spectrum with a color-coding for visualization purposes. STOCSY has the advantage over traditional 2D NMR correlation methods such as TOCSY[46,47] as positive correlations can be seen between resonances with no spin-coupling, so all peaks from the same molecule can, in principle, be identified assuming that there is not extensive overlap of peaks with those of other compounds. This overlap problem is acute in samples containing multiple saccharides, polyols or glucuronides as may often be seen in biofluids. Analysis of lower or negative (but still statistically significant) correlations has the potential to allow molecules in the same chemical or metabolic pathways to be determined. This method has recently been used to characterize structural pathway connectivities of drug metabolites,[43] and analyze complex spectral data from liquid chromatography−NMR spectroscopy data.[44] It has also been extended to visualize diffusion-edited NMR data,[41] to enhance biomarker detection by applying heteronuclear $^{1}H-^{31}P$ STOCSY to MAS NMR spectra of liver and gut samples,[39,42] and $^{1}H-^{19}F$ STOCSY has been utilized for fluorinated drug metabolism studies.[45]

The main aims of the present study were to investigate the efficiency of the STOCSY approach for assignment of NMR spectral peaks in a chemically reacting system, and the development of JRES-STOCSY to improve information recovery from heavily overlapped signals and spin-systems. We have exemplified the approach using the degradation of a model drug AG, (*S*)-α-methyl phenylacetyl AG (MPG), which is a stuctural analogue of (*S*)-ibuprofen AG, the statistical analysis of spectra collected over the time-course of the acyl migration reactions being designed to extract connectivity information through correlation of signals from the different isomers as they are formed or degraded.

EXPERIMENTAL SECTION

Materials. 3-[2,2,3,3-$^{2}H_4$]Trimethylsilyl propionate sodium salt (TSP), sodium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from Sigma-Aldrich Company, Ltd.

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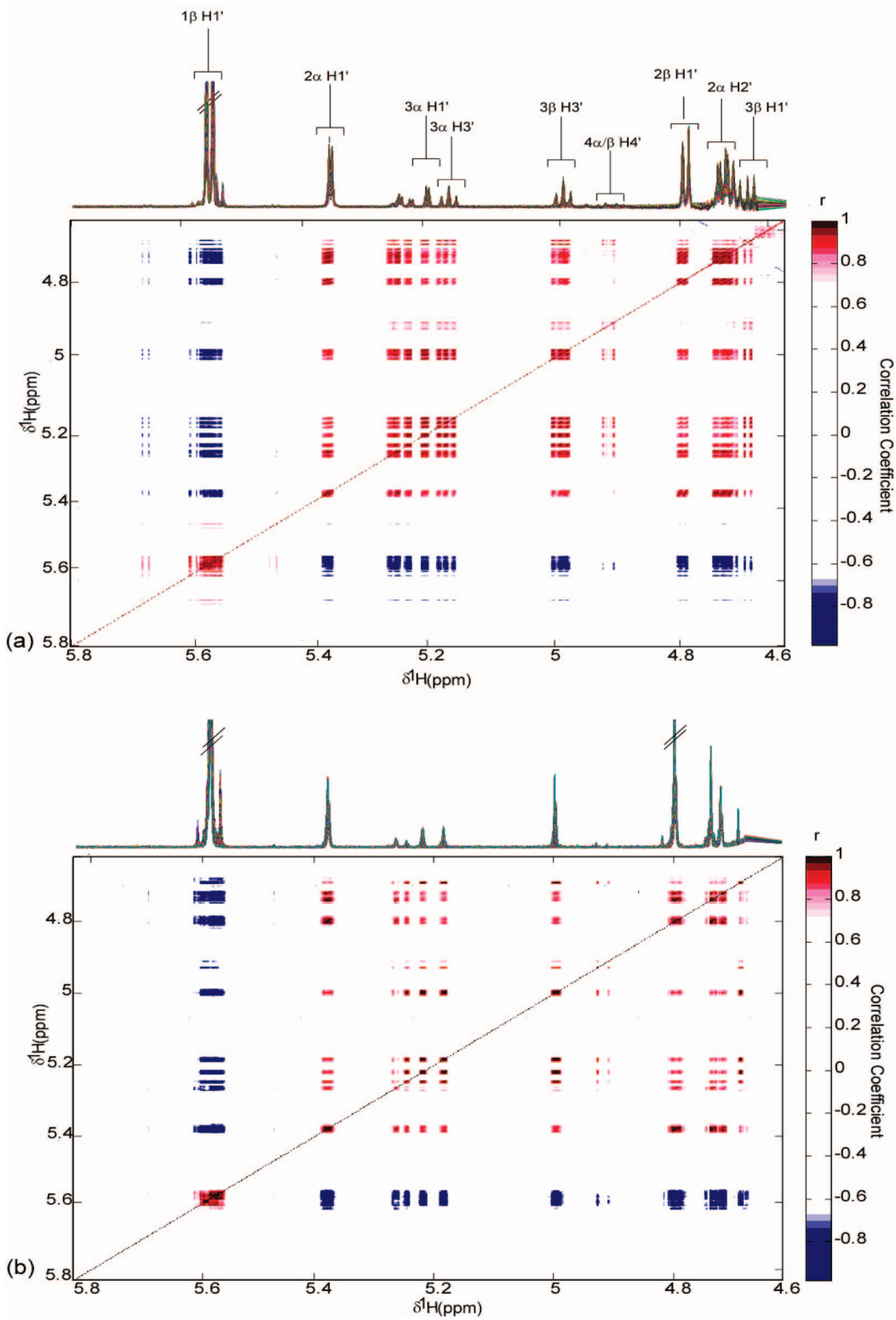

Figure 4. (a) Localized pseudo 3D correlation matrix of ^{1}H 1D NMR spectra showing correlations between all isomers. (b) Localized pseudo 3D matrix of ^{1}H 1D JRES projections showing correlations between all isomers. 1βH1 etc. denotes the H1′ proton resonance of the 1-β-isomer.

(Gillingham, Dorset, U.K.). HPLC−NMR grade deuterium oxide ($^{2}H_2O$) was obtained from Goss Scientific Instruments (Essex, U.K.).

Chemical Synthesis of 1-β-*O*-Acyl (*S*)-α-methyl Phenylacetyl Glucuronide (MPG). A model drug glucuronide [1-β-*O*-acyl (*S*)-α-methyl phenylacetyl glucuronide] was chemically synthesized using a variation of the selective acylation method as recently documented.[13,48,49] The method involves alkylation of

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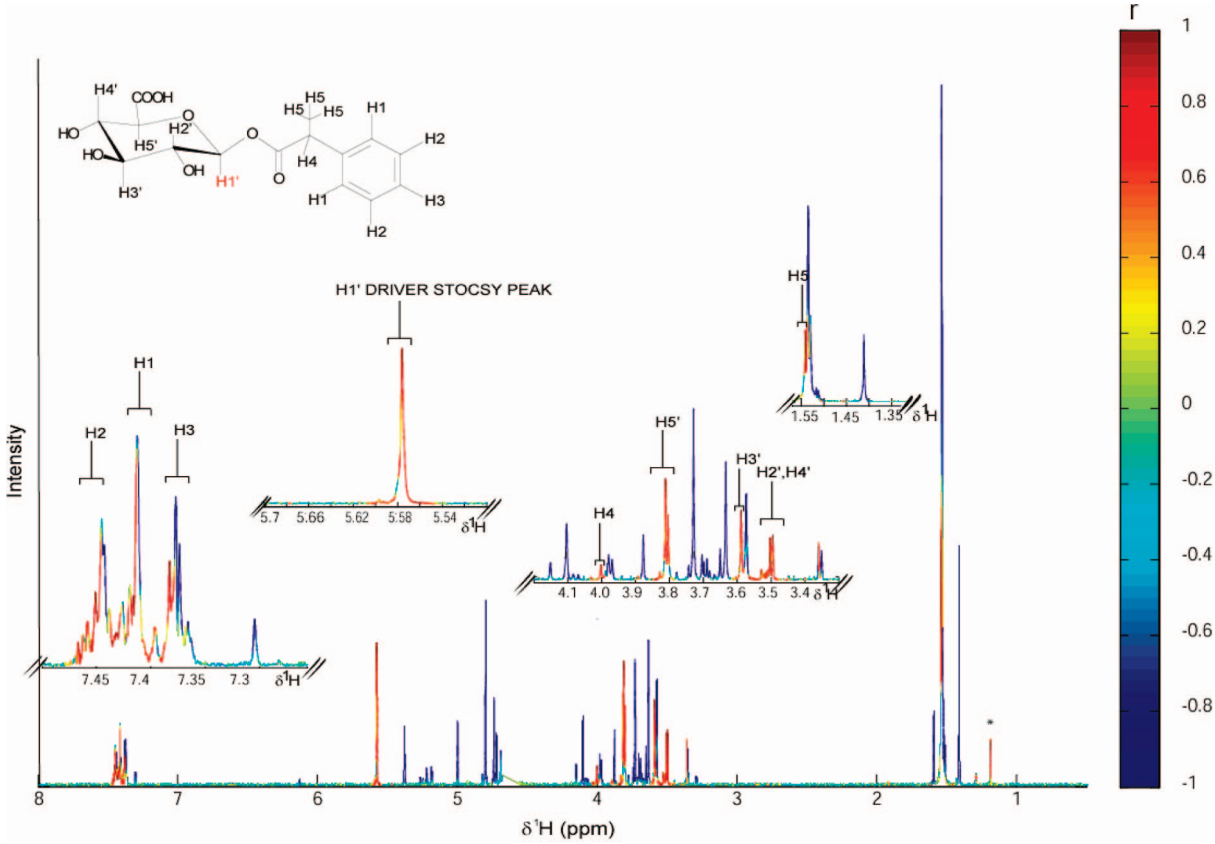

Figure 5. STOCSY plot calculated from the covariance to the data point corresponding to the maximum of the (*S*)-α-methyl phenylacetyl AG H1′ proton resonance at δ 5.58 (0−4.5 h) (*minor AG contaminant).

benzyl glucuronate[49] with racemic α-methyl phenylacetic acid following activation of the carboxylic acid using Carpino's uronium reagent *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and 1,4-diazabicyclo[2.2.2]octane (DABCO). The diastereoisomeric benzyl esters were separated by chromatography, and then the (*S*)-benzyl ester was deprotected by catalytic dehydrogenation to afford the title compound as the highly pure 1-β isomer.

^{1}H NMR Spectroscopy. All NMR experiments were carried out at 310 K on a Bruker Avance 800 spectrometer equipped with a 5 mm *z*-gradient triple-resonance inverse (TXI) ^{1}H/^{13}C/^{15}N cryoprobe at 800.32 MHz. The ^{1}H 1D NMR spectra were recorded using a standard water peak presaturation pulse sequence[36] (to suppress the residual H_2O signal), and 16 scans were acquired with a spectral width of 16025.5 Hz and a relaxation delay (RD) of 2 s, with a total acquisition time of 1.55 min per spectrum. The ^{1}H−^{1}H 2D JRES spectra were acquired using the standard pulse sequence RD−90°−$t_{1/2}$−180°−$t_{1/2}$−ACQ (t_2), where t_1 is an increment delay and t_2 is the acquisition time. Four scans per increment for 20 increments were acquired into 16 K data points, with a spectral width of 7684.4 Hz in the F2 domain (from t_2) and 25 Hz in the F1 dimension (from t_1) and a RD of 2 s. Each spectrum was acquired in 8.73 min. Once the acquisition parameters had been optimized using a standard sample, 550 μL of 100 mM sodium phosphate buffer in ^2H_2O (pD 7.4) was added quickly to 1.5 mg of MPG. The sample was transferred to a 5 mm NMR tube containing 50 μL of TSP (0.5 mg/mL in ^2H_2O), and an automated program was set up to acquire 103 loops of the 1D and 2D experiments, which resulted in a total experiment time of 17.65 h. An exponential apodization function corresponding to a line-broadening factor of 0.3 Hz was applied to the 1D spectral FIDs. The JRES data were zero-filled to 64 K (F2) and 128 (F1) points prior to Fourier transformation, and a sine-bell apodization function was applied followed by conventional tilting and symmetrization.

Spectral Processing and STOCSY Calculations. JRES skyline projections on to the F2 axis were calculated for each experiment and treated as 1D NMR spectra for STOCSY analysis purposes. These data were imported into MATLAB 7.4.0 using in-house software (MetaSpectra: O. Cloarec, and T. Ebbels Imperial College London), and scaled to a constant value for the TSP peak intensity at δ 0.0. The region containing the small water residual peak between δ 4.56 and 4.68 was removed to eliminate the variation in water suppression. For calculation of 2D STOCSY correlation matrices, each spectrum was imported into 4800 data points with a computer point resolution of 1.6 Hz prior to data analysis.

Correlation coefficients were calculated for data points corresponding to peaks in the JRES projection selected for each of the eight positional isomers. A pseudo-2D NMR matrix was plotted showing the covariance between all the variables in the spectrum. Pseudo-1D NMR plots were made by back-projecting the correlation coefficients between a selected variable and all the other variables of the spectrum on to the final NMR spectrum acquired after equilibration for 18 h. The plots were color-coded to represent the degree of correlation between the selected variable and all the other variables in the spectrum.

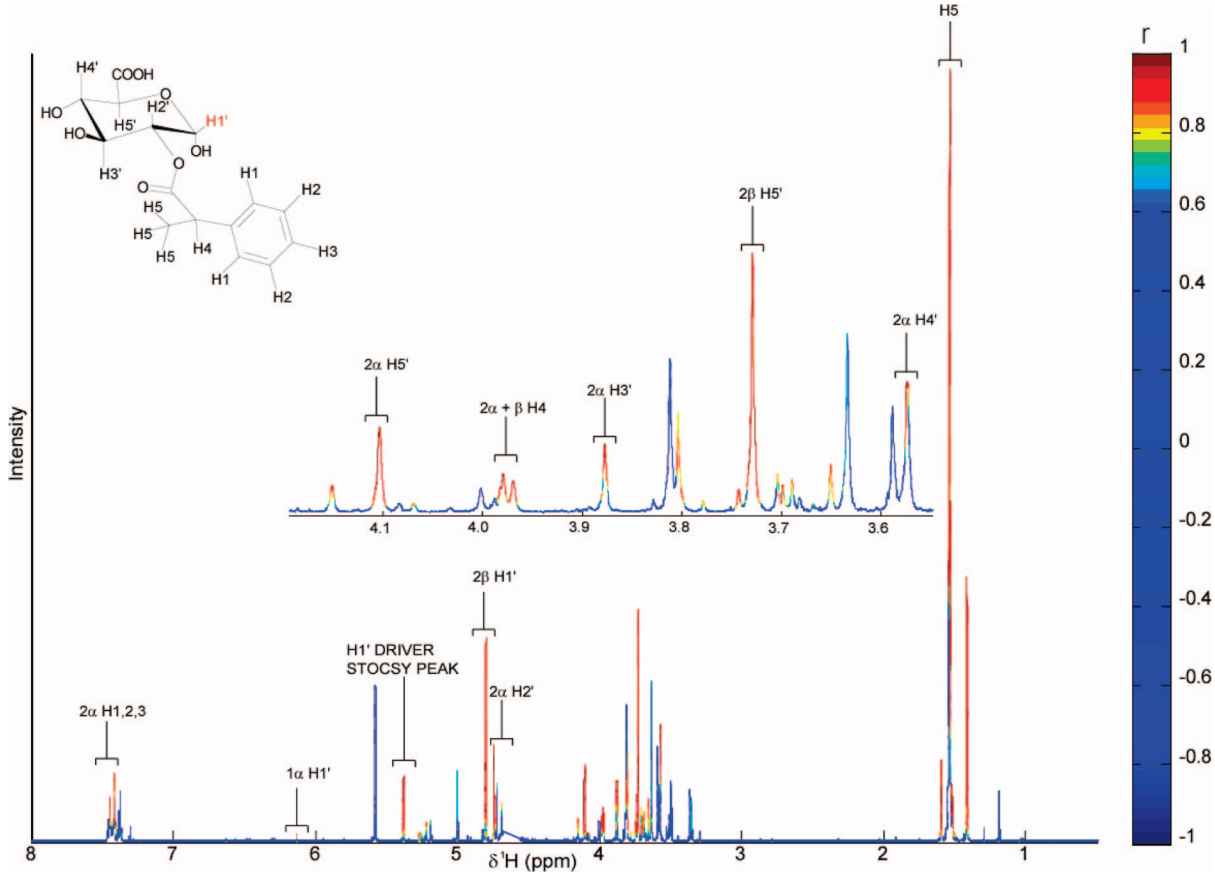

Figure 6. STOCSY plot calculated from the covariance to the data point corresponding to the maximum of the (*S*)-α-methyl phenylacetic acid AG 2α H1′ proton resonance at δ 5.40 (4.5−9 h).

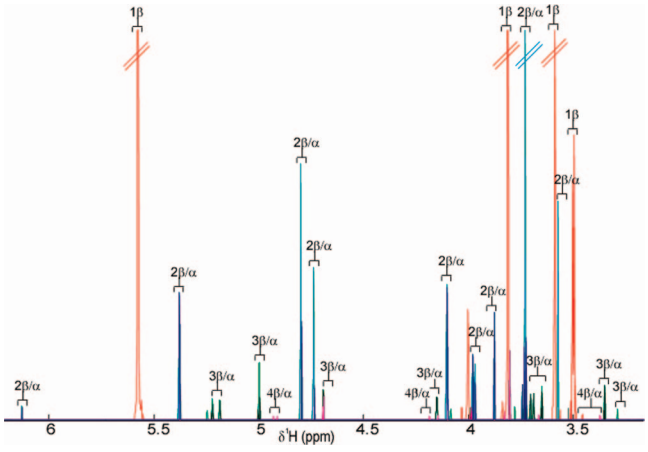

Figure 7. A statistically reconstructed 1D NMR spectrum showing 8 overlaid coefficient plots for the median spectra of each isomer. Correlation coefficients >0.9 are shown only, allowing each isomer to be isolated. The color scheme is chosen to define each isomer separately.

RESULTS AND DISCUSSION

1D and 2D NMR Spectroscopy. A ^{1}H 1D NMR spectrum of MPG is shown in Figure 1(a). Figures 1(b) and 1(c) show the ^{1}H−^{1}H JRES NMR spectrum in the form of a 1D skyline projection and 2D contour plot respectively. The contour plot displays the chemical shifts and coupling constants in two orthogonal frequency domains, with the skyline projections displaying these data as a 1D spectrum of singlets. Even though these spectra show resonances from only one compound, there is some overlap in the proton resonances in the anomeric region and for the glucuronide ring protons at ∼δ 3.5. However, it is clear that the JRES data is of great value for assigning resonances and multiplicity in these regions.

The full advantage of acquiring the JRES NMR spectra can be seen in Figure 2, which compares a series of ^{1}H 1D NMR spectra with the ^{1}H 1D JRES skyline projections at the same time-points for the δ 3.2−6.2 region of the glucuronide ring protons. The complexity of the ^{1}H 1D spectra in (a) illustrates the difficulty in monitoring a dynamic system when there are a large number of overlapped spin-coupled multiplet resonances. The skyline projections seen in (b) allow the peaks to be more resolved, and thus the change in peak heights can be seen more clearly. The JRES experiment is based on the spin−echo pulse sequence, and peak intensities are dependent on the proton T_2 values as well as concentration. In addition, these data are often heavily weighted, particularly in the *J*-coupling (F1 axis), using functions such as the shifted sine-bell, or Lorentzian−Gaussian transformation, of which the pseudoecho is a special case. Thus both of these effects can, in principle, cause errors in peak intensities that might affect any subsequent correlation analysis. However, in the reacting glucuronide system as studied herein, where all of the species involved are either isomers or closely related structurally, proton T_2 values likely to be similar.

STOCSY experiments are always made less effective by peak overlap as independent signals lower the covariance. The degree of ^{1}H NMR spectral dispersion seen in Figure 2 could normally only be achieved with unattainably high NMR magnetic field strengths

as illustrated in Figure 3. Figure 3(a) shows a system of two overlapped doublets separated by a chemical shift of 1 Hz, each with coupling constants of $J = 7$ Hz. If this result had been achieved at a frequency of 800 MHz, in order to separate them into two completely resolved doublets by a spacing of 1 Hz, as shown in Figure 3(c), i.e. chemical shift difference of 8 Hz, the NMR experiment would need to be carried out on a magnet with $8 \times B_0$ field strength (ca. 150 T operating at 6.4 GHz) which is not possible with current technology. For comparison, the JRES projection is shown in Figure 3(b), and because of the removal of *J*-coupling splittings, the 1 Hz separation is easily seen. A similar argument applies to partially overlapped triplets, where the hypothetical increase in observation frequency to cause complete separation of the two multiplets with a chemical shift difference of 1 Hz, by 1 Hz, would be a factor of 15, as shown in Figures 3(d) to (f). Therefore, the effect of projecting 1D skyline NMR spectra from 2D JRES data produces an apparent or virtual spectral dispersion that is not achievable using conventional NMR spectrometers, and this is most useful in STOCSY analysis of skyline intensity covariance to demonstrate intramolecular connectivity.

STOCSY Analysis of NMR Data. The STOCSY analysis was carried out across the full set of 103 time course 1D NMR experiments to show correlations between spectral peak intensities in the reacting system. Figure 4(a) shows a correlation matrix of the ^{1}H 1D spectra and can be interpreted as a pseudo-3D NMR spectrum (the axes hold information about the coupling constants, chemical shift and time domain). Each peak from the NMR spectra can be seen on the diagonal line, and correlations between peak intensities appear as diagonal peaks similar to a standard TOCSY ^{1}H NMR spectrum. A corresponding correlation matrix of the ^{1}H 1D JRES projections can be seen in (b) illustrating the decrease of spectral complexity, and the increase in the spectral dispersion when compared to (a). High correlations are seen for peaks arising from the same positional isomer and also between mutarotating isomers since this reaction is fast[50] compared to the degradation rate.[28]

Due to the sequential nature of the isomerization kinetics, not all peaks vary strongly in intensity over the whole time course, and so a "time slice" variant was applied to the kinetic STOCSY approach and ^{1}H 1D K-JRES-STOCSY analyses were carried out over 4 time intervals, 0−4.5, 4.5−9, 9−13.5 and 13.5−18 h. By analyzing each interval separately, it was possible to identify the time-period which best discriminated the individual isomers and taking into consideration only the highest correlation coefficient, this permitted the identification of the NMR peaks arising from each individual isomer. Within each interval a "driver" peak (the peak from which a data point is chosen at its apex to initiate the statistical analysis) was selected for the isomer of interest, and correlations to this peak were calculated using spectra acquired in the time-course interval and back-projected onto the final spectrum at the end of the experiment where all isomers are present. A 1D K-JRES-STOCSY plot calculated using the 1D JRES projection NMR spectra acquired in the 0−4.5 h time-period is shown in Figure 5. Correlation coefficients were calculated for correlations between a data point belonging to the H1′ proton of the 1-β isomer and the rest of the data points in the matrix over the 4.5 h, and the coefficients for each data-point were back-projected onto the final (18 h) NMR spectrum. It can be clearly seen that the highest spectral correlations ($r > 0.9$) with the peak of interest ($\delta = 5.58$, H1′ 1-β driver) all arise from the 1-β isomer, and clearly differentiate the peaks of this isomer from those of the other isomers. (Nomenclature: 2-β H1′ refers to the proton at the C-1 position on the glucuronide ring of the 2-β positional isomer etc.) Correlations are not seen between the 1-β and 1-α anomers as formation of the latter is not by mutarotation but by positional isomerization, with the 1-α isomer being formed through a back-reaction from the 2-α isomer.

Targeted 1D K-JRES-STOCSY analyses were carried out to identify peaks from the other isomers within the spectral sets where the peaks were at their maximum intensity for that isomer, in order to maximize variation for distinguishing each isomer. A K-JRES-STOCSY plot for experiments acquired in the 4.5−9 h time-period, showing correlation to the 2-α H1′ proton, is shown in Figure 6. A strong correlation ($r > 0.9$) is seen with the other protons in the 2-α isomer, as well as those from the 2-β isomer. As seen in Scheme 1, the 2-α isomer can undergo mutarotation to the 2-β isomer, hydrolysis to the aglycon, and also acyl migration to form the 3-α and 1-α isomers, and apart from hydrolysis all are reversible reactions. Correlations to the 3-α and 1-α isomers have high coefficients of $r = 0.88$ and $r = 0.72$, while anticorrelations are seen to the 1-β isomer $r = -0.92$.

A K-JRES-STOCSY analysis carried out on the 13.5−18 h time-period using the same driver peak gives different correlation coefficients from those calculated in the 0−4.5 h analysis. The 2-α isomer now appears anticorrelated to the 3-α and 1-α isomers, $r = -0.45$ and $r = -0.12$ correspondingly, while correlation to the 1-β isomer is higher than previously observed at $r = 0.19$. This can be explained by the rate of formation of the 2-α isomer, which increases rapidly from 0 to 10 h and then slows from 10 h onward, and the 2-α/2-β isomer concentrations then decline. At 10 h the 1-β isomer concentration was still decreasing, and the 3-α and 1-α isomers were increasing, generating anticorrelations to the 1-β signal intensities.

Correlations can also be investigated over the time-course of the entire 18 h experiment. For each of the 8 isomers, an edited median spectrum was generated such that only points in the spectrum which corresponded to a threshold correlation coefficient $r > 0.9$ were displayed, and the other points were set to 0. An overlay of these 8 edited spectra is shown in Figure 7, illustrating how the use of a correlation coefficient threshold can help differentiate specific isomer resonances.

The acquisition of JRES spectra decreases the spectral complexity and facilitates the generation of each STOCSY correlation plot by increasing the spectral dispersion. Therefore the considerable peak overlap is eliminated, and furthermore, good signal-to-noise ratios are obtained in a relatively short period of time. The JRES-STOCSY method also allows structural information about protons from the same molecule that are not spin−spin coupled to be detected, and allows connectivities to be established in the dynamic pathway. This technique could also be used effectively to help assign metabolite identities in complex samples such as biofluids, where there is considerable overlap of multiple compound peaks throughout the NMR spectral regions. The JRES technique has already been shown to be valuable for analysis of complex mixtures of endogenous metabolites,[34–38] and so the combination JRES-STOCSY technique will be particularly useful for metabonomic/metabolomic applications.

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By obtaining multiple NMR spectra, in this case ^{1}H 1D skyline projections of the JRES data, it is possible to capture the reaction processes, and with the addition of STOCSY analysis, the pathway of these processes can be defined, and mixtures of isomers can be assigned. This type of analysis is particularly useful when dealing with reacting systems, operating at the minute time-scale which is conducive to rapid sequential measurements on highly sensitive cryoprobes. For more rapid chemical reactions, simple one-shot ^{1}H NMR spectra obtainable in as little as one second could be used. The application of the K-STOCSY approach here has been observed with transacylation reactions of the 1-β-*O*-AGs, and correlations between isomers have been shown to differ as the experimental time increases, but this technique could also be applied to other dynamic or enzyme-catalyzed metabolic systems.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was posted on 5/10/08. An error in the labeling in Scheme 1 was noted and corrected. The paper was reposted on 5/29/08.

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