Mixture Analysis in Viscous Solvents by NMR Spin Diffusion Spectroscopy: ViscY. Application to High- and Low-Polarity Organic Compounds Dissolved in Sulfolane/Water and Sulfolane/DMSO-d6 Blends

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Mixture Analysis in Viscous Solvents by NMR spin diffusion spectroscopY: *ViscY*. Application to High- and Low-polarity Organic Compounds dissolved in Sulfolane/Water and Sulfolane/DMSO-d_6 blends

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ABSTRACT: The sulfolane/water and sulfolane/DMSO-d_6 binary NMR solvents are reported for the first time for the individualization of mixture components by spin diffusion, when molecular tumbling is slow due to solvent viscosity, thus strongly favouring magnetization transfer by dipolar cross relaxation. All ^{1}H nuclei resonances within the same molecule tend to correlate in a 2D NOESY spectrum, opening the way to mixture analysis. Till now, analysis of organic compounds by NMR spin diffusion in viscous solvents involved ^{1}H, ^{13}C, ^{15}N and ^{19}F.We offer a new way to analyse mixtures by considering ^{31}P nuclei as chemical shift markers. We report the individualization of four polar dipeptides and of four non-polar phosphorus-containing compounds respectively dissolved in sulfolane/water and sulfolane/DMSO-d_6 solvents blends by means of homonuclear selective 1D and 2D ^{1}H experiments and a heteronuclear 2D ^{1}H-^{31}P HSQC-NOESY experiment by taking advantage from spin diffusion. The name *ViscY* is proposed to refer to the class of all NMR spectroscopy experiments that rely on viscous solvents for mixture analysis.

Over the last few years, identification of organic compounds in mixture has become a key research subject. Cosmetics, fine chemical and pharmaceutical companies always seek for innovative solutions for the structural characterization of organic compounds of low molecular weight in mixture, obtained either by synthesis (products of interest or by-products), or by extraction from natural or biotechnological resources, that are easy and cheap to implement and in conformity with current regulations. Analytical techniques for the determination of the structure of unknown organic compounds within mixtures rely often on the coupling of chromatographic and spectroscopic methods, such as high performance liquid chromatography (HPLC) coupled with NMR (LC-SPE-NMR) or/and mass spectrometry (LC-MS). [1] When physical separation is not possible, NMR must be performed on mixtures as they are. Being able to assign each NMR peak to a specific molecule reduces the need for chromatographic separation and thus would greatly increase the efficiency of natural and synthetic chemists.

So far, analysis of individual mixture components by NMR without any physical separation has been explored in a limited number of ways: i) In the 2D DOSY experiment, NMR signals are labelled according to the value of the translational diffusion coefficient (D) of each molecule in mixture. [2-3] However, this experiment provides only limited resolution along the D axis. NMR signals from structurally close molecules may be therefore difficult to group according to their parent compound, even though experimental tips may enhance the resolution of the 2D DOSY experiment: addition of chromatographic phases to the sample, interaction of analytes with soluble polymers or lanthanide shift reagents, analyte inclusion in micelles. [4-9] ii) Multiquantum spectroscopy combined (or not) with broadband homonuclear decoupling, sparse sampling, pure shift, ultrafast data acquisition or tensor decomposition methods may be relevant for individualizing molecules in mixture. [10-15] iii) The use of viscous solvents (or solvent blends) provides an interesting analytical approach in the study of complex mixtures. These solvents lower the tumbling rate of small and medium sized molecules in solution since the value of the molecular overall correlation time τ_c depends on the medium viscosity according to the microviscosity theory of Gierer and Wirtz. [16]. The corresponding longitudinal cross relaxation regime thus favours spin diffusion. As a result, molecules fall in the negative nuclear Overhauser effect (NOE) regime and their resonances can be grouped according to their ability to share magnetization by intramolecular spin diffusion. All resonances of ^{1}H nuclei within the same molecule tend to correlate together in a 2D NOESY spectrum, therefore giving access to the individual ^{1}H NMR spectra of the mixture components. The initial idea was implemented in 1981 [17] by means of a perfluorinated polymer as solvent, which was re-examined in 2008; the use of supercooled water to modulate the spin relaxation dynamics of small metabolites was reported in 2012 by the same team. [18] Our team published original results in the field of mixture analysis by NMR through the use of glycerol and glycerol carbonate (in 2011), DMSO/glycerol (in 2016), DMSO/water (in 2017), and sucrose solution and agarose gel (in 2019) as viscous solvents for the creation of spin diffusion conditions in ^{1}H and ^{19}F NMR. [19-23] Henceforth, we propose the name *ViscY* (the second syllable has to be pronounced as in "whisky") for referring to all NMR methods in which *Visc*ous solvents or solvent blends promote the individualization of mixture components by NMR spin diffusion spectroscop*Y*.

To date, only few viscous solvents have been described in the literature for the study of high- and low-polarity compounds within mixtures. In this context, the present work focusses on the assessment of two viscous sulfolane-based solvent blends, namely sulfolane/water and sulfolane/DMSO-d_6, in the individual NMR characterization of four highly-polar, structurally close dipeptides: Leu-Val, Leu-Tyr, Gly-Tyr and Ala-Tyr and of four lowly-polar phosphorous-containing compounds by means of spin diffusion in homonuclear selective 1D ^{1}H

NOESY, selective 2D ^{1}H NOESY experiments and a heteronuclear 2D ^{1}H-^{31}P HSQC-NOESY.

Sulfolane is a cheap, colourless, non-reactive dipolar aprotic solvent with high chemical and thermal stability and unusual solvent properties. It is made of globular molecules and presents a moderately high dielectric constant (ε = 43.4 at 300 K) [24] and a high dipole moment (μ = 4.80 D). [25] Sulfolane is completely miscible with water and other polar and aromatic organic solvents. First produced and patented in the 1940s, it has been used in a wide variety of industrial production processes for aromatic solvent extraction, petroleum production and refining. [26] It is also employed in polymer technology as solvent, plasticizer, and synthesis medium. It is a solvent of choice for acid-catalysed reactions at high temperatures such as Friedel-Crafts acylations and Bischler-Napieralski condensations. It has also been used as a solvent for oxidations, nitrations, chemical rearrangements, phosphonylations, and condensation reactions. [27] Although sulfolane presents a higher melting point (300.5 K) than water (273 K) and DMSO-d_6 (292 K), its melting point can be drastically lowered by adding water or DMSO-d_6. [28] For instance, melting points of the sulfolane/water and sulfolane/DMSO-d_6 solvent blends respectively reach 256 K and 238 K after adding 50% of water and 30% of DMSO-d_6 by volume. This observation opens a way to work at or below room temperature, which is especially appropriate for thermally unstable compounds within mixtures. Spin diffusion may therefore take place on a wide range of temperatures, from room to sub-zero temperatures due to the viscosity increase of both these solvent blends upon temperature lowering. [29] Nevertheless, the viscosity of the sulfolane-based solvents at room temperature remains sufficiently low, even if pure sulfolane reveals a higher viscosity (η = 10.29 cP at 303 K) [30] than the ones of water (η = 0.898 cP at 298 K) and of DMSO-d_6 (η = 2.007 cP at 298 K), [31] so that samples are prepared and transferred into the NMR tube without any difficulty, in contrast with highly viscous solvents such as glycerol (η = 934 cP at 298 K) and glycerol carbonate (η = 85.4 cP at 298 K). [32] Nonetheless, the major experimental drawback of considering these sulfolane-based solvent blends lies in the strong ^{1}H NMR signals of non-deuterated sulfolane and water that force their suppression. This is easily managed by means of selective excitation and detection pulses included in a double pulsed field gradient spin echo (DPFGSE) sequence. [33] In addition, the high amount of added DMSO-d_6 (compared to 10% of D_2O in volume in sulfolane/H_2O/D_2O solvent blend) facilitates the use of spectrometer tools such as automatic field-locking and shimming, like for any other usual NMR solvent.

The sulfolane/water solvent blend is appropriate for the study of high-polarity compounds whereas the viscous sulfolane/DMSO-d_6 binary solvent is adapted to the investigation of compounds of low-polarity. Mid-sized polar and lowly-polar molecules necessitate a low amount of water or DMSO-d_6 in sulfolane whereas smaller or more flexible molecules require more water or DMSO-d_6 for driving spin diffusion under temperature control from room temperature to sub-zero temperatures.

The choice of an optimal operating temperature results from a compromise between spectral resolution and intensity of NOESY cross peaks between nuclei that are not close enough to show a NOE signal in a low viscosity medium. A temperature reduction favours spin diffusion but also reduces peak height through line broadening due to a more efficient transverse relaxation. Sample cooling is therefore required if the NOESY spectrum shows positive NOE responses (diagonal and off-diagonal peaks of opposite signs). Depending on the complexity of the mixtures, the analysis of ^{1}H NMR spectra may become impossible owing to the overlapping of ^{1}H resonances. A conventional remedy to this issue consists in the spreading of the spectroscopic information along a second axis that encodes chemical shifts of nuclei other than ^{1}H. [20-22] This approach to mixture analysis is exemplified in sulfolane/DMSO-d_6 solvent blend by means of the 2D ^{1}H-^{31}P HSQC-NOESY experiment providing ^{1}H/^{31}P chemical shift lists for the mixture of low polarity compounds.

EXPERIMENTAL SECTION

Chemical reagents. DMSO-d_6 and D_2O were purchased from Eurisotop (Gif-surYvette, France). Leu-Val, Leu-Tyr, Gly-Tyr and Ala-Tyr were purchased from TCI Europe (Zwijndrecht, Belgium). Sulfolane, dicyclohexyl(4-(*N*,*N*-dimethylamino)phenyl)phosphine and exo-phenyl Kwon [2.2.1] bicyclic phosphine were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Allyltriphenylphosphonium bromide and (methoxymethyl)triphenylphosphonium chloride were purchased from Acros Organics (Geel, Belgium). All peptides and phosphorus-based compounds had 95% or higher purity and were dissolved at a concentration of 20 mM respectively in sulfolane/H_2O/D_2O (5:4:1 v/v/v) and in sulfolane/DMSO-d_6 (7:3 v/v).

NMR Spectroscopy: The NMR experiments on the dipeptide and phopshorus-based compounds test mixtures were performed on a Bruker Avance AVIII-500 NMR spectrometer equipped with a 5 mm BBFO+ probe using the Bruker TOPSPIN Software (Rheinstetten, Germany). Static field gradient pulses were generated by a 10 A amplifier, so that the sample is submitted to a nominal 0.535 Tm^{-1} gradient. Gradient pulses were followed by a 200 µs recovery delay. Temperature was controlled by a Bruker variable temperature (BVT) unit supplied with chilled air produced by a Bruker cooling unit (BCU-Xtreme).

Dipeptide mixture spectra were calibrated so that the tyrosine Hα proton resonances appeared at δ 7.00. Additional NMR data acquisition and processing parameters for Figures 1-4 and 6-8 are described in the Supporting Information (SI) file.

RESULTS AND DISCUSSION

Leu-Val, Leu-Tyr, Gly-Tyr and Ala-Tyr mixture in sulfolane/H_2O/D_2O (5:4:1 v/v/v)

These four dipeptides do not present any differentiation in the DOSY spectrum when dissolved in pure water owing to their similar molecular weight and shape (Figure S1 in the Supporting Information (SI)). The use of viscous solvents such as sulfolane/water has opened the way to other strategies than DOSY, namely *ViscY*, relying on homo- and heteronuclear NOESY-based spin diffusion experiments for mixture analysis. The main experimental pitfall of our analytical approach is the mandatory elimination of the strong ^{1}H signals of sulfolane and water (see Figure 1a) for avoiding to drastically obscure solute signals, since deuterated sulfolane would be too expensive to produce and full deuterated water would result in the chemical exchange between the deuterium nuclei of the latter and the amide protons of the dipeptides. The elimination of solvent signals was obtained by means of selective pulses when included in a DPFGSE sequence (Figures 1b, 1e and 1c, 1f). [33] The selective pulses invert the equilibrium magnetization of the nuclei of

interest and leave untouched the one of the solvent nuclei. For this aim, resonance inversion in the two frequency bands on either side of sulfolane signals was successfully achieved by means of two consecutive band-selective pulses. The spectrum in Figure 1b demonstrates the quality of solvent suppression that was obtained by band-selective detection (see pulse sequence in Figure 1e). All solute signals in the solvent frequency band are eliminated as well. Nevertheless, this is not an issue for the band-selective 2D NOESY experiment, provided that at least one resonance per analyte is preserved. This resonance can correlate with all others along the F_1 axis, including those located in the solvent spectral region by taking advantage from spin diffusion.

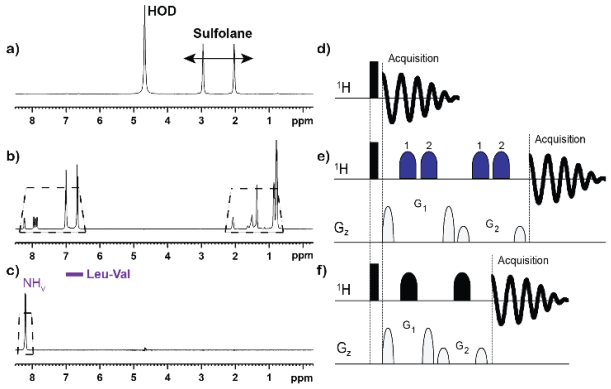

Figure 1. 1D proton spectra and corresponding NMR pulse sequence of the dipeptide test mixture: Leu-Val, Leu-Tyr, Gly-Tyr and Ala-Tyr (20 mM) dissolved in sulfolane/H_2O/D_2O (5:4:1 v/v/v), at 258 K, at 500 MHz (^{1}H). a, d) Non-selective excitation and detection. b, e) Selective detection of two resonance bands. The 4 ms I-BURP-2 pulses cover 1250 Hz (dotted trapezium). The "1" and "2" labels respectively indicate their application to the high and low chemical shift regions. c, f) Selective excitation of the valine amide proton doublet of Leu–Val (dotted trapezium) using a 30 ms, 1% truncated, 180° Gaussian pulse.

Since the operating temperature is a crucial parameter in spin diffusion experiments due to its direct influence on solvent viscosity and as a result on overall rotational correlation times τ_C, [16, 19] we have searched for the optimal temperature at which NOESY cross peaks were positive (negative NOE enhancements, slow motion regime), well-resolved, and as intense as possible between nuclei that were not supposed to be close enough to present a NOE in low viscous medium. The optimized temperature of 258 K has been determined by means of band-selective detection NOESY experiments (see Figure 2a, Figures S2: amide proton region NOESY spectra at 298, 288, 278, 268 and 258 K and S3: full NOESY spectrum at 258 K in SI). The use of viscous sulfolane/water solvent mixture clearly allows full intramolecular magnetization transfer through spin diffusion, detected over distances of > 14 Å within each very small and flexible dipeptide. On the contrary, the NOESY spectrum recorded in water at 298 K reveals fewer NOE cross peaks, all of opposite sign (positive NOE enhancements, fast motion regime, see Figure 2b, full NOESY spectrum in Figure S4 in SI). As a result, the grouping of proton resonances is possible under *ViscY* conditions, making the individualization of the mixture components possible since the chemical shift pattern of each dipeptide is predictable. The individualization of the four dipeptides in water would have required the concomitant use of NOESY and TOCSY (and COSY) experiments, according to the well-established resonance assignment strategy. [34]

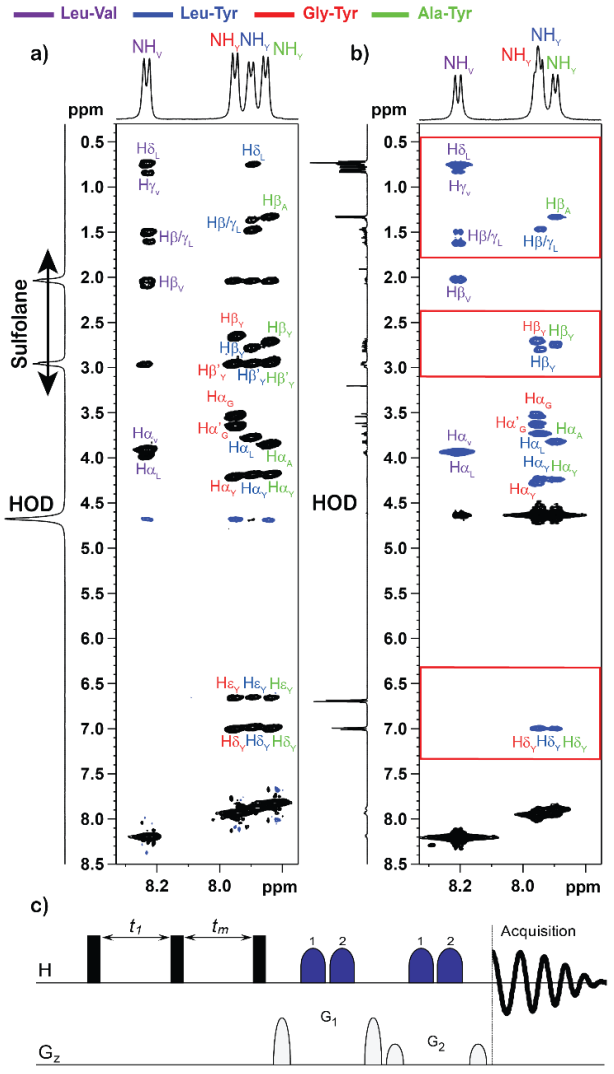

Figure 2. a) Amide proton region of band-selective detection 2D NOESY spectrum of the dipeptide test mixture (20 mM) dissolved in sulfolane/H_2O/D_2O (5:4:1 v/v/v), mixing time (t_m) = 1 s, at 258 K, at 500 MHz (^{1}H), using the pulse sequence in part c. b) Amide proton region of 2D NOESY spectrum of the same dipeptide test mixture (20 mM) dissolved in H_2O/D_2O (9:1 v/v), t_m = 1 s, at 298 K, at 500 MHz (^{1}H), using the noesyesgpph pulse sequence. The red frames correspond to spectral regions in which water has a major effect on the number and sign of NOESY cross peaks.

In band-selective detection 2D NOESY spectra, H_α and H_β proton resonances are only detected in F_1 because they are suppressed in F_2 owing to their proximity with solvent signals. Therefore, it seemed pertinent to record additional structural information on mixture components by detecting these H_α and H_β resonances in F_2 during signal acquisition. For this purpose, an appropriate set of resolved proton resonances was selectively excited by means of 1D selective-NOESY experiments which are composed of a double pulse field gradient block for the multiplet selective excitation of the resonance of interest followed by a mixing period (Figure 3f). The latter includes two wideband adiabatic inversion pulses framed by gradient pulses in order to avoid the resurgence of strong sulfolane and water signals during the mixing time that arise from longitudinal relaxation.

[33, 35] This analytical approach involving the detection of only resonances of interest may turn out to be relevant in case of intense proton resonance overlapping especially for complex mixtures.

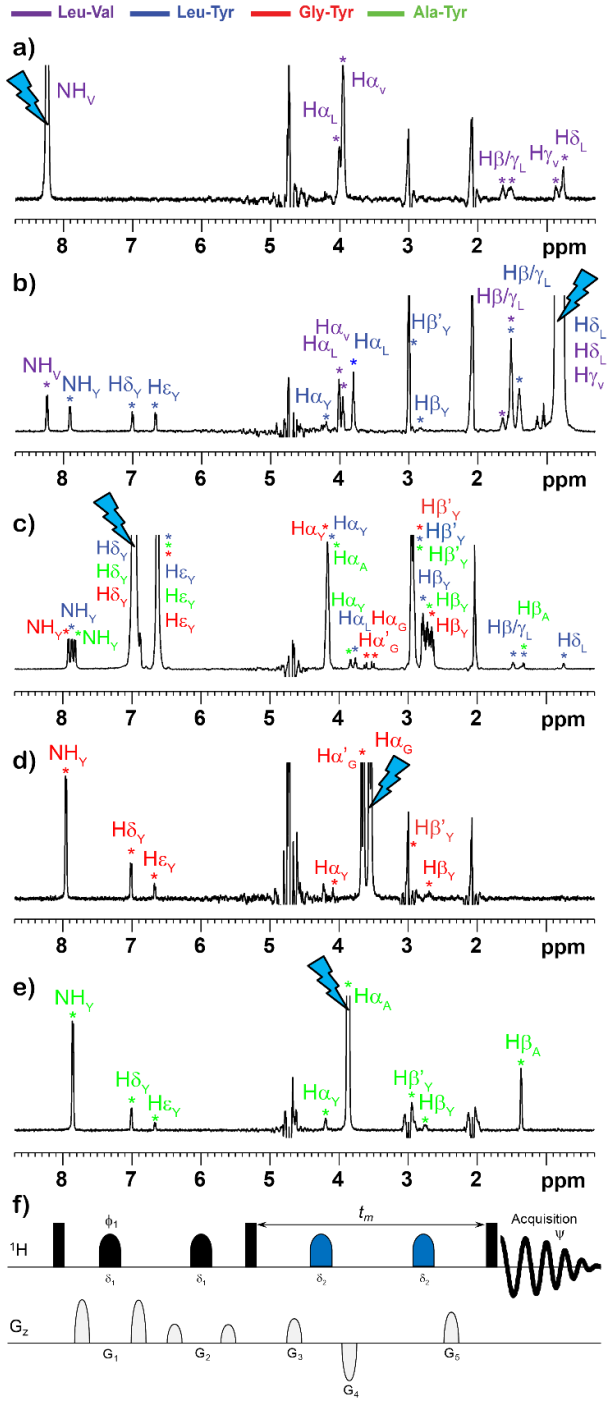

Figure 3. Multiplet selective excitation 1D ^{1}H NOESY spectra of the dipeptide test mixture (20 mM) dissolved in sulfolane/H_2O/D_2O (5:4:1 v/v/v) (a, b, c, d and e), t_m = 1 s, at 258 K, at 500 MHz (^{1}H). The initial selective inversion pulses excite: a) the NH_V(LV), b) the $H\delta_L$(LY)/$H\delta_L$(LV)/$H\gamma_V$(LV), $H\delta_Y$(LY)/$H\delta_Y$(GY)/$H\delta_Y$(AY) d) the $H\alpha_G$(GY) and e) the $H\alpha_A$(AY) proton resonances. f) Pulse sequence: $\varphi_1 = x, y, -x, -y$, $\psi = x, -x$.

Figure 3 clearly proves that all dipeptides are differentiated by spin diffusion in sulfolane/water solution by means of a suitable set of selectively excited proton resonances. Indeed, the selective excitation of the NH amide proton at δ 8.23 leads to a magnetization transfer exclusively with the protons of the Leu-Val dipeptide because the tyrosine Hδ/Hε proton resonances do not appear in the 1D NOESY spectrum (Figure 3a). The selective excitation of the side chain Hδ and Hγ protons (between 0.72 and 0.90 ppm) reveals a magnetization exchange with all protons of the two Leu-Val and Leu-Tyr dipeptides (Figure 3b). By comparison with both 1D NOESY spectra in Figures 3a and 3b, an entire proton assignment of Leu-Tyr is produced. The selective excitation of the aromatic Hδ/Hε protons of Leu-Tyr, Gly-Tyr and Ala-Tyr at 7.00 ppm shows all the proton resonances of Leu-Tyr, Gly-Tyr and Ala-Tyr (Figure 3c). In order to differentiate all proton resonances from Gly-Tyr and Ala-Tyr, one of the Hα protons at δ 3.53 has been selectively excited (Figure 3d). Figure 3d clearly shows the transfer of the glycine Hα magnetization over all protons of Gly-Tyr because of the absence of the side chain proton ($H\beta_A/\beta_L/\gamma_L/\delta_L$) resonances of Leu-Tyr and Ala-Tyr. Another way to distinguish Gly-Tyr from Ala-Tyr has been to selectively excite the $H\alpha_A$ resonance at 3.86 ppm which unveils a magnetization exchange exclusively with all protons of Ala-Tyr (Figure 3e).

A faster experimental strategy than the recording of five appropriate selective 1D NOESY spectra was to focus on the close NH amide resonances and to resort to a F_1 band selective F_1 decoupled 2D NOESY experiment [36-37] since they appear in the same frequency band and they do not arise from scalarly coupled nuclei (see Figure 4). Only the amide protons provided the transverse magnetization after the initial band selective excitation step. Afterward, the doublet amide proton signal of each dipeptide was collapsed into a singlet of higher intensity by means of a selective echo followed by a nonselective echo in the middle of t_1, both framed by gradient pulses. The mixing time block, including wideband inversion pulses for thrawting the reintroduction of sulfolane and water signals, let spin diffusion spread along the proton network of each molecule. As a result, carrying out this NOESY experiment in sulfolane/water solvent blend makes possible to assign almost all proton resonances of Leu-Val, Leu-Tyr, Gly-Tyr and Ala-Tyr (except Tyr Hβ' resonances hidden by sulfolane signals) by taking advantage from spin diffusion under viscous conditions.

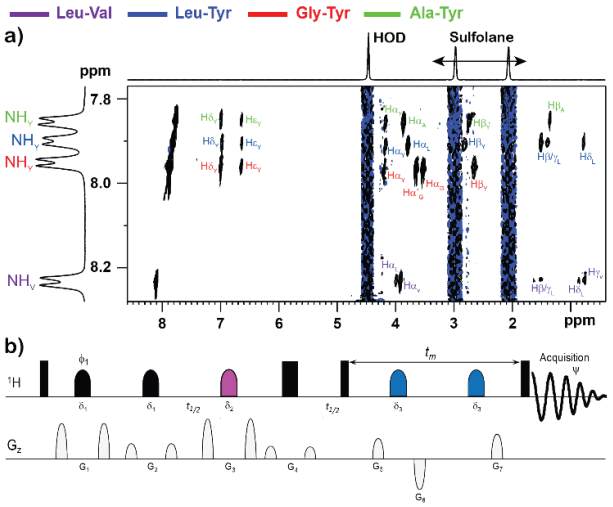

Figure 4. a) F_1 band selective F_1 decoupled 2D NOESY spectrum of the dipeptide test mixture (20 mM) dissolved in sulfolane/H_2O/D_2O (5:4:1 v/v/v), t_m = 1 s, at 258 K, at 500 MHz (^{1}H), b) Pulse sequence: $\varphi 1 = x, y, -x, -y$, $\psi = x, -x$. The initial selective

180° pulses (δ_1 = 4 ms) had a Gaussian shape and were applied to the four NH amide proton resonances.

Dicyclohexyl(4-(*N*,*N*-di!methylamino)phenyl) phosphine, exophenyl Kwon [2.2.1] bicyclic phosphine, allyltriphenylphosphonium bromide and (methoxymethyl)triphenylphosphonium chloride mixture in sulfolane/DMSO-d_6 (7:3 v/v)

The simplification of mixture analysis by means of heteronuclear chemical shift resonance labelling has been extended to ^{31}P NMR spectroscopy. Phosphorus is a chemical element that is present in many chemical synthesis intermediates and biological molecules of interest. It has a natural abundance of 100% and reveals moderate relaxation times that provides sharp lines. [38] The broad range of ^{31}P chemical shift covers about 2000 ppm and is thus one order of magnitude larger than that of carbon, and two orders of magnitude larger than that of proton, what should facilitate the individualization of phosphorus-containing molecules within mixtures. [39] In 2017, we reported that ^{19}F nuclei, similarly to ^{1}H nuclei, participated to spin diffusion along the molecular spin network of a difluorinated compound in the viscous DMSO-d_6/H_2O solvent, at 238 K and 500 MHz (^{1}H). [21] However, unlike ^{19}F nucleus which has a magnetogyric ratio close to the one of ^{1}H, the ^{31}P nucleus reveals positive HOE (heteronuclea NOE) that tends towards zero in the spin diffusion limit. [40] As a result, phosphorus-based compounds will never produce negative HOE when long molecular overall correlation times are observed. A remedy to this experimental issue is to consider ^{31}P nuclei as chemical shift markers and to induce magnetization transfer along the intramolecular proton network by means of the 2D ^{1}H-^{31}P HSQC-NOESY experiment. Hereby, we describe for the first time a promising approach in the individualization of four mixed phosphorus-based compounds dissolved in sulfolane/DMSO-d_6 (7:3 v/v) solvent blend under spin diffusion conditions by means of homonuclear selective 1D NOESY, selective 2D NOESY experiments and a heteronuclear 2D HSQC-NOESY experiment.

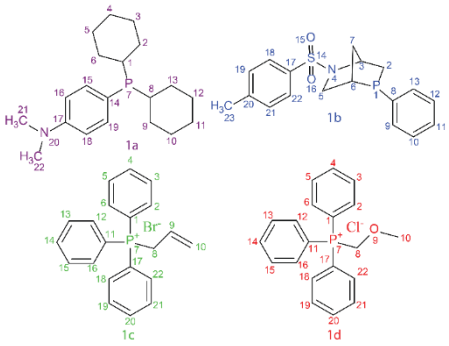

Figure 5. Chemical structures of the four phosphorus-containing compounds within mixture: 1a) dicyclohexyl (4-(*N*, *N*-dimethylamino)phenyl) phosphine, 1b) exophenyl Kwon [2.2.1] bicyclic phosphine, 1c) allyltriphenylphosphonium bromide and 1d) (methoxymethyl)triphenylphosphonium chloride.

As for the dipeptide test mixture dissolved in water, the four mixed phosphorus-containing compounds do not reveal any differentiation by translational diffusion in pure DMSO-d_6, due to their similar molecular mass (Figure S5 in SI). This observation prompted us to investigate this mixture dissolved in a sulfolane-based solvent blend compatible with low polarity molecules such as the sulfolane/DMSO-d_6 binary solvent. The resolving power of NOESY-based spin diffusion experiments should allow us to individualize each mixture component. Once again, the main experimental drawback in considering non-perdeuterated sulfolane solvent was the mandatory suppression of its strong ^{1}H signals (see the conventional 1D ^{1}H spectrum in Figure S6a in SI). The elimination of these signals was also achieved by selective pulses involved in an excitation sculpting block (Figures S6b and c in SI). [33]

The impact of temperature on intramolecular spin diffusion has been discussed in the previous section. Similarly, we have determined the temperature that provides the best compromise between efficient ^{1}H spin diffusion and relevant ^{1}H and ^{31}P spectral resolution by means of band-selective detection 2D ^{1}H NOESY, at 500 MHz (^{1}H) and ^{1}H-decoupled ^{31}P experiments at 298, 288, 278, 268, 258 and 248 K, at 202.46 MHz (^{31}P). Positive NOE enhancements (negative NOESY cross peaks, fast motion regime) are still observed at 278 K (Figure S7 in SI). Spin diffusion starts to be active at 268 K for all molecules. A complete magnetization transfer along proton network of each compound is clearly visible at 248 K. However, compound 1a reveals too much active T_2 transverse relaxation, responsible for ^{31}P resonance line broadening (Figure S10 in SI). Consequently, the optimized temperature of 258 K has been retained for further studies because it offered efficient ^{1}H spin diffusion (negative NOE enhancements, positive NOESY cross peaks, slow motion regime) and suitable ^{1}H and ^{31}P spectral resolution.

The band-selective detection 2D NOESY spectrum at 258 K clearly revealed four positive NOE cross-peak patterns, each corresponding to a single mixture component (see Figure 6 and full NOESY spectrum in Figure S8 in SI). The pattern identification of 1a is guided by the distinctive aromatic protons $H_{16,18}$ at 6.72 ppm (see Figure 6c for individual 1D ^{1}H spectrum of 1a vertically extracted from 2D NOESY). The pattern of 1b is distinguished by means of the tropanic ring H_3 at 4.45 ppm (individual 1D ^{1}H spectrum of 1b in Figure 6d). The proton H_{10} at 5.40 ppm make it possible to distinguish the proton pattern of 1c (individual 1D ^{1}H spectrum of 1c in Figure 6e). The proton resonance H_8 at 5.54 ppm is a relevant starting point for identifying the complete resonance pattern of 1d (individual 1D ^{1}H spectrum of 1d in Figure 6f). We have clearly observed that the sulfolane/DMSO-d_6 solvent allows a complete intramolecular magnetization transfer by spin diffusion, detected over distances greater than 13 Å within each phosphorus-containing compound, in part owing to the stiffness of the cycloalkyl and aromatic moieties. In contrast, when the conventional 2D NOESY spectrum is recorded in neat DMSO-d_6 at 298 K, the four mixed phophorus-based molecules rapidly reorient and present a positive NOE regime thus preventing spin diffusion to be observed (see Figure 6b and full NOESY spectrum in Figure S9 in SI).

Due to resonance peaks overlapping in the 1D and 2D spectra of the four mixed phosphorus-containing compounds, it was quite challenging to unambiguously assign each proton resonance to a specific molecule within their mixture. The multiplet selective excitation 1D NOESY experiment, previously described in this work for the dipeptide test mixture, should address this issue by grouping proton resonances belonging to the same compound. The main experimental pitfall regarding the reintroduction of strong ^{1}H non-perdeuterated sulfolane signals during the mixing time of the NOESY block is still treated by means of wideband pulses framed by gradient pulses. [33] Choosing an appropriate set of selectively excited proton resonances

allows to obtain the individual 1D ^{1}H spectrum of each mixed molecule in the viscous sulfolane/DMSO-d_6 (7:3 v/v) binary solvent by taking profit from spin diffusion. Figure 7 shows the selective excitation 1D NOESY spectrum of each mixed phosphorus-based compound. In particular, the individualization of 1a is carried out by the selective excitation of the aromatic protons $H_{16,18}$ at 6.72 ppm (Figure 7a). All proton resonances of 1b are observed by means of the selective excitation of the tropic ring proton H_{2a} at 1.96 ppm. Interestingly, spin diffusion is able to connect signals from the two aromatic moieties of 1b (Figure 7b). Selective excitations of the ethylene protons H_{10} at 5.40 ppm and the methyl group H_{10} at 3.53 ppm make it possible respectively to group together all proton resonances of 1c and 1d (Figures 7c and d).

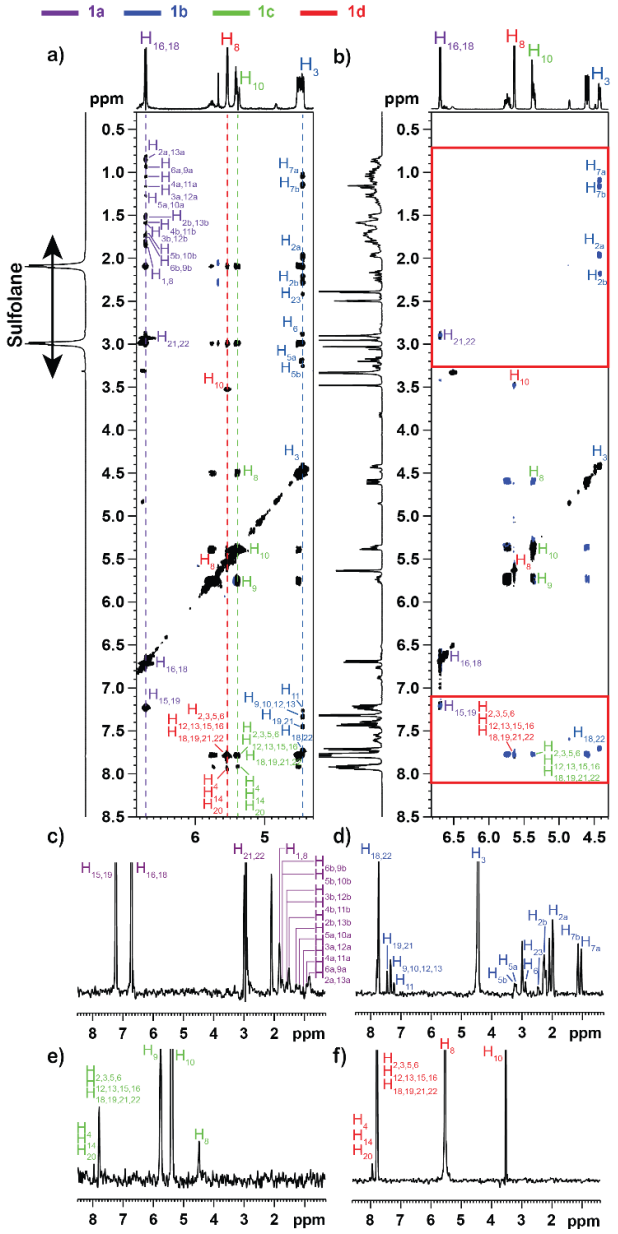

Figure 6. a) Low-field proton region of the band-selective detection 2D NOESY spectrum of the phosphorus-based compound test mixture (20 mM) dissolved in sulfolane/DMSO-d_6 (7:3 v/v), t_m = 1 s, using the pulse sequence in Figure 2c, at 258 K, at 500 MHz (^{1}H). ^{1}H vertical slices extracted from the 2D ^{1}H NOESY at 6.72 ppm (c, 1a, $H_{16,18}$, purple dotted line), at 4.45 ppm (d, 1b, H_3, blue dotted line), at 5.40 ppm (e, 1c, H_{10}, green dotted line), and at 5.54 ppm (f, 1d, H_8, red dotted line). b) Low-field proton region of the 2D NOESY spectrum of the same phosphorus-based compound test mixture dissolved in neat DMSO-d_6, t_m = 1 s, at 298 K, at 500 MHz (^{1}H). The red frames correspond to spectral regions of interest in which DMSO-d_6 has a major effect on the number and sign of observable NOESY cross peaks.

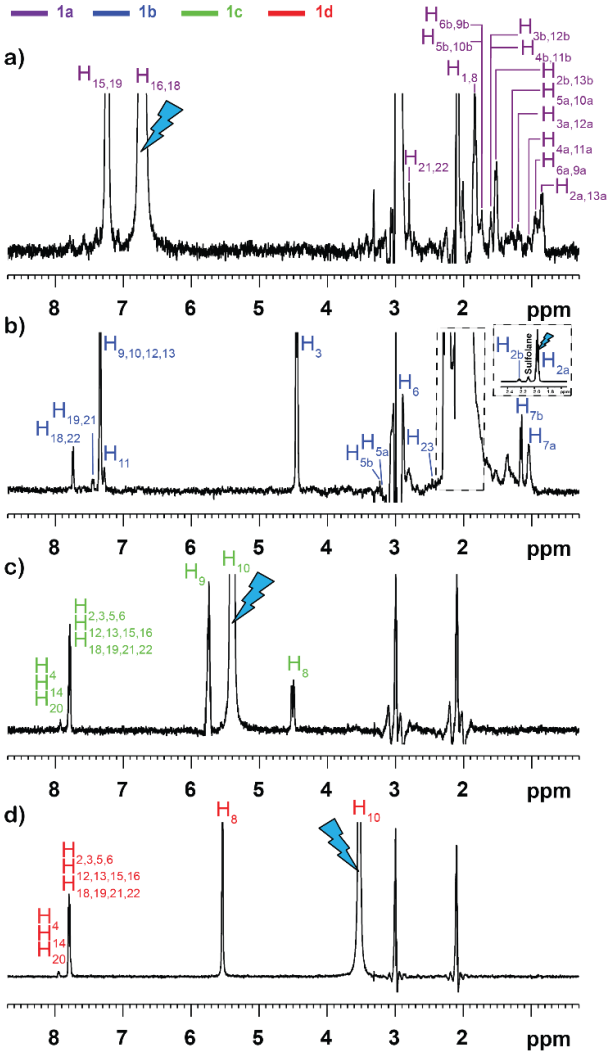

Figure 7. Multiplet selective excitation 1D ^{1}H NOESY spectra of the phosphorus-based compound test mixture (20 mM) dissolved in sulfolane/DMSO-d_6 (7:3 v/v) (a, b, c and d), t_m = 1 s, at 258 K, at 500 MHz (^{1}H). Pulse sequence (Figure 3f). The initial selective inversion pulses excite: a) the $H_{16,18}$ (1a), b) the H_{2a} (1b), c) the H_{10} (1c) and d) the H_{10} (1d) proton resonances.

The multiplet selective excitation 1D ^{1}H NOESY experiment has demonstrated that grouping proton resonances belonging to the same compound within mixture thus allowing its individualization is possible by taking benefit from spin diffusion. However, in the study of other more complex mixtures, ^{1}H spectral overlap may occur. As a result, compounds of interest may not present resolved proton resonances, thus preventing the use of 1D and 2D ^{1}H selective NOESY experiments.

In such cases, the broad chemical shift range of ^{31}P may prove to be relevant. By coupling 2D HSQC and NOESY experiments, a complete proton spectrum should be obtained for each component within mixture starting only from one single phosphorus resonance in the indirect dimension.

The 2D ^{1}H-^{31}P HSQC-NOESY spectrum of the phosphorus-based compound test mixture dissolved in sulfolane/DMSO-d_6 (7:3 v/v) solvent blend recorded at 258 K is drawn in Figure 8. Under these *ViscY* operating conditions, all the protons of each molecule are able to correlate with all other protons by spin diffusion after having marked the phosphorous chemical shift in F_1. The selection of four slices through ^{31}P resonances at - 2.00 (Figure 8b, 1a, purple row), - 17.34 (Figure 8c, 1b, blue row), 21.01 (Figure 8d, 1c, green row), and 17.32 ppm (Figure 8e, 1d, red row) allows to produce, respectively, four complete proton spectra corresponding to 1a, 1b, 1c and 1d. These four spectra are compared to the previous selective 1D NOESY spectra (Figures 8b, b'; c, c'; d, d'; e, e') and the proton resonance patterns are similar, as expected. As a result, we have demonstrated for the first time the capability to individualize phosphorous-containing compounds within mixture by means of the 2D ^{1}H-^{31}P HSQC-NOESY experiment under viscous conditions.

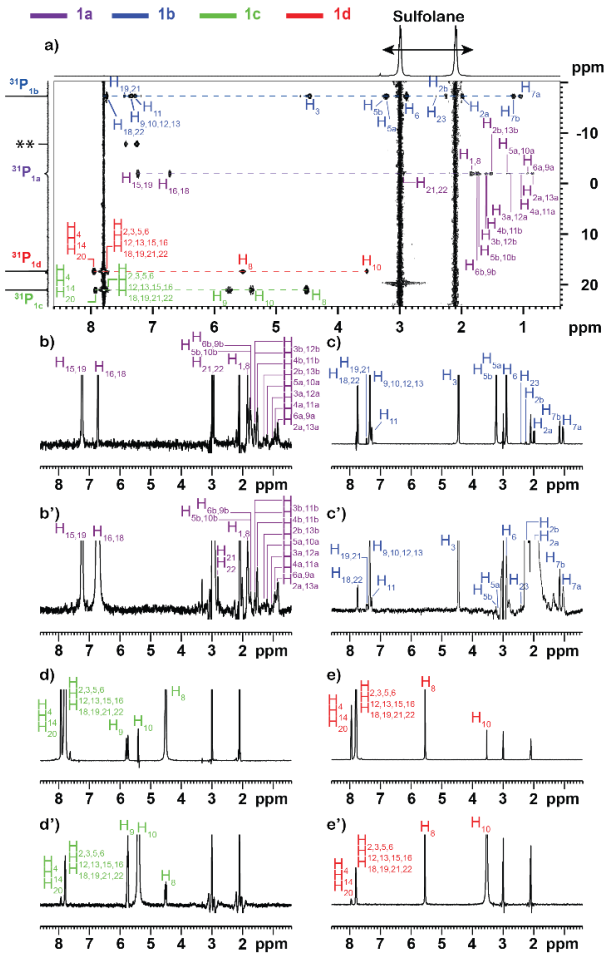

Figure 8. a) 2D ^{1}H-^{31}P HSQC-NOESY spectrum of the phosphorus-based compound test mixture (20 mM) dissolved in sulfolane/DMSO-d_6 (7:3 v/v), t_m = 1 s, at 258 K, at 500 MHz (^{1}H). Comparison of four ^{31}P horizontal slices extracted from the 2D ^{1}H-^{31}P HSQC-NOESY at - 2.00 (b, b', 1a, purple dotted line), - 17.34 (c, c', 1b, blue dotted line), 21.01 (d, d', 1c, green dotted line), and 17.32 ppm (e, e', 1d, red dotted line) with the 1D selective NOESY spectra (selection of, b') the $H_{16,18}$, c') the H_{2a}, d') the H_{10} and e') the H_{10} protons resonances). ** ~~Impurety~~Impurity.

CONCLUSIONS

We have established for the first time that the use of sulfolane/water and sulfolane/DMSO-d_6 as viscous binary solvents makes possible the individualization of respectively high- and low-polarity components within complex mixtures, by taking advantage from NMR spin diffusion.

The component individualization within the Leu-Val, Leu-Tyr, Gly-Tyr and Ala-Tyr mixture and within the dicyclohexyl(4-(*N*,*N*-dimethylamino)phenyl) phosphine, exophenyl Kwon [2.2.1] bicyclic phosphine, allyltriphenylphosphonium bromide and (methoxymethyl)triphenylphosphonium chloride mixture respectively in sulfolane/H_2O/D_2O (5:4:1 v/v/v) and sulfolane/DMSO-d_6 (7:3 v/v) solvents has been achieved at 258 K by homonuclear selective 1D ^{1}H NOESY, selective 2D ^{1}H NOESY and heteronuclear 2D ^{1}H-^{31}P HSQC-NOESY experiments. The latter offers a new analytical way, never described up to now under viscous conditions, in order to extract the individual spectrum of phosphorus-containing compounds within mixture by taking advantage of the relevant spectrum readability ~~of~~ brought by the broad ^{31}P chemical shift range.

We have pointed out that viscous sulfolane-based solvents present valuable advantages compared to previously described highly viscous solvents such as glycerol and glycerol carbonate.[19] The spin diffusion is active in a wider temperature range, compatible with thermally fragile compounds, and the NMR sample preparation is easy. The viscous sulfolane/water solvent blend is recommended for the study of polar compounds within mixture whereas sulfolane/DMSO-d_6 is more dedicated to the investigation of mixed low-polarity compounds. Mid-sized high- and low-polarity molecules require a low amount of water or DMSO-d_6 in sulfolane whereas smaller or more flexible molecules necessitate more water or DMSO-d_6 in sulfolane until 50% (v/v) for driving spin diffusion from room to sub-zero temperatures.

Future investigations in the field of mixture analysis following the *ViscY* approach will cover the study of other polar and apolar mixtures made of small-sized molecules for assessing spin diffusion power of viscous sulfolane-based binary solvents.

ASSOCIATED CONTENT

Supporting Information

Relevant NMR data acquisition and processing parameters used for the study and additional 2D ^{1}H NOESY and 1D ^{31}P spectra (PDF file). The Supporting Information is available free of charge on the ACS Publications website.

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The authors declare no competing financial interests.

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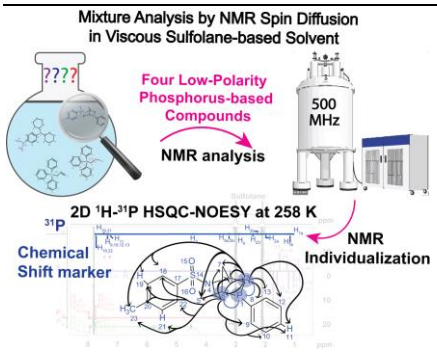
Mixture Analysis by NMR Spin Diffusion
in Viscous Sulfolane-based Solvent
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Four Low-Polarity
Phosphorus-based
Compounds
NMR analysis
500
MHz
2D ¹H-³¹P HSQC-NOESY at 258 K
³¹P
Chemical
Shift marker
NMR
Individualization