Quantitative Monitoring of Solid Phase Organic Reactions by High-Resolution Magic Angle Spinning NMR Spectroscopy

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Three possible high-resolution magic angle spinning (HR MAS) NMR experiments to quantitatively monitor a solid phase supported Horner−Emmons reaction are presented. In the first experiment we follow the solid phase reaction in deuterated solvent directly in the NMR rotor. The second quantification is done by reconditioning of a few milligrams of resin from an undefined reaction vessel by washing, drying, and reswelling in deuterated solvent, and the evaluation of the amount of resin bound structures by comparing to an external standard. The third experiment represents the first analytical quantification of resin-bound structures without any sample preparation, except the transfer of resin−solvent suspension (large excess of reagents in protonated dimethylformamide) from the reaction vessel to the NMR rotor.

Introduction

Solid-phase organic chemistry (SPOC)[1,2] has definite advantages over homogeneous phase organic chemistry, since the use of large excesses of reagents and subsequent purification by washing are possible, thereby greatly simplifying both automation and workup procedures. Nevertheless, this technique of modern organic chemistry still suffers from a lack of rapid analytical techniques for efficient reaction monitoring. Especially in the case of combinatorial chemistry, this is an important drawback, as one needs to identify reaction conditions that work equally well in a multitude of chemical contexts. The recent success of SPOC and its wide application in combinatorial chemistry therefore have resulted in an increased effort toward the development of efficient analytical tools suitable for the analysis of resin bound structures, avoiding the tedious and time-consuming procedure of cleave-and-analysis. For more complex reactions, the technique of high-resolution magic angle spinning NMR (HR MAS)[3−8] is emerging as a new tool that finally could solve the analytical difficulties of SPOC. In a case study treating the developmental stage of a combinatorial chemistry library, Luo et al.[9] clearly pointed out its decisive advantages at more elaborate steps of the reaction, with the possibility of identifying every single proton and carbon. With exactly the same pulse sequences as in high-resolution liquid NMR, time requirements and resulting resolution can equally be compared. Even further, we recently showed that the insoluble nature of the polymer can be used as an analytical advantage, as it allows exploitation of the differential diffusion properties of attached molecules and solvent in order to suppress the latter, making an analysis of resins in protonated solvents feasible.[10]

Whereas the above-described techniques all have shown their potential in the identification of resin-bound molecules, easy quantification is a further step needed in the reaction monitoring and optimization. Here, we demonstrate three possible HR MAS experiments to monitor, quantify, and optimize a solid-phase chemical reaction without cleaving the molecules from the resin. Their relative disadvantages and merits are discussed. In the first experiment, we follow a complete reaction in the rotor under magic angle spinning conditions while using DMF-d_7 as a solvent. Reaction progress is observed by comparing the decreasing signal of the added reagent in solution to the increasing signal of the resin-bound reaction product. Because the rotor represents unrealistic reaction conditions, we run in a second experiment the chemical reaction under realistic conditions in a normal vessel, and sample the resin at different time events. After washing, drying, and swelling of these samples in deuterated solvent, NMR spectra are acquired, and the amount of polymer-linked product is determined by comparison to a soluble standard added to the rotor. Finally, in a third experiment, we demonstrate the feasibility of following directly ongoing chemical reactions under standard conditions (using protonated solvent and excess reagents in nonspecific reaction vessels) by simple transfer of a resin suspension from the reaction vessel to the MAS NMR rotor. As we do not remove the protonated solvent anymore, we have to suppress their NMR signals by the recently described differential diffusion filter.[10] Reaction progress is followed by comparing

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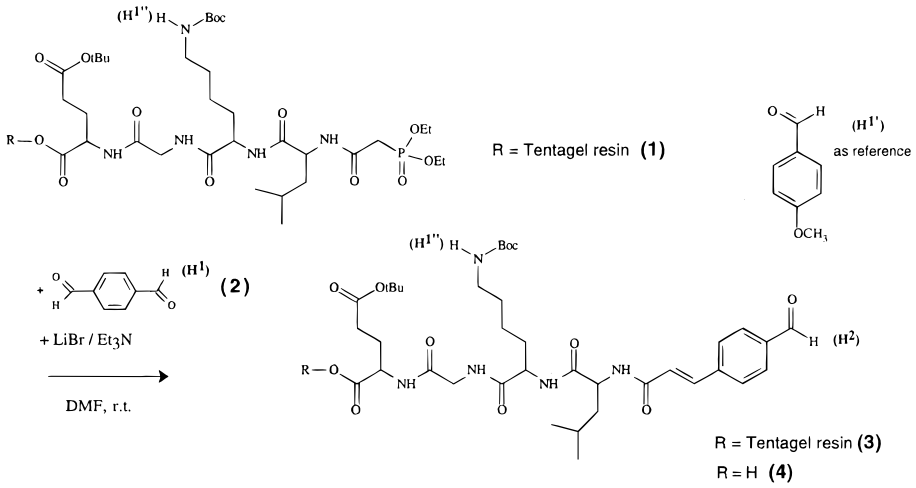
Scheme 1



an increasing signal unique to the polymer-linked product with a proton signal that both the polymer-linked starting material and product have in common.

Results and Discussion

The reaction monitoring is demonstrated on a Horner−Emmons reaction on polyethylene-grafted polystyrene/1% divinylbenzene[11] (Scheme 1), where a phosphonodiester linked to the polymer through a tetrapeptide (**1**) is reacted with an excess of soluble terephthalaldehyde (**2**) to produce the polymer bound monoaldehyde (**3**).

In our first experiment, the polymer bound starting material (**1**) was loaded directly into the rotor, swollen in DMF-d_7, and the reaction was started by adding consecutively terephthalaldehyde and Et_3N/LiBr, both dissolved in DMF-d_7. Proton spectra recorded at appropriate time intervals allowed to follow the reaction progress by comparing the integrals of the signal H^1 of the soluble dialdehyde reagent (**2**) and the integral of the resin-attached reaction product (**3**, H^2 proton). The amount of polymer-linked product (**3**) *y* is calculated as *x*I2/(I2 + I1/2) where *x* is the initial amount of added dialdehyde (5 equiv with respect to the theoretically present reaction sites on the resin) and I1 and I2 are the peak volumes of the signals of the two H^1 protons of the soluble dialdehyde (**2**) and the H^2 proton of polymer-linked reaction product (**3**), respectively. In the first assay, 5 equiv of dialdehyde (**2**) and 10 equiv of Et_3N/LiBr were used. After 20 h and 50% conversion of the polymer-bound starting material (**1**), the reaction kinetic diagram leveled off and no further progress could be detected (Figure 1a). However, this plateau does not imply that all available sites on the resin have actually reacted, as the absence of both reagents or catalyst, the appearance of moisture or neutralizing agents in the reaction medium might prevent reaction completion. By adding 5 equiv of Et_3N/LiBr at two different time events, the reaction restarted and 65% and 80% conversion to **3** were obtained. Dimer formation by a potential inter-resin second Horner−Emmons reaction of the polymer-linked monoaldehyde **3** with nonreacted reaction sites (**1**) on the same resin was not detected (2D-HR MAS spectra of **3**, TOF-PDMS and HPLC of **4** after cleavage from the resin).

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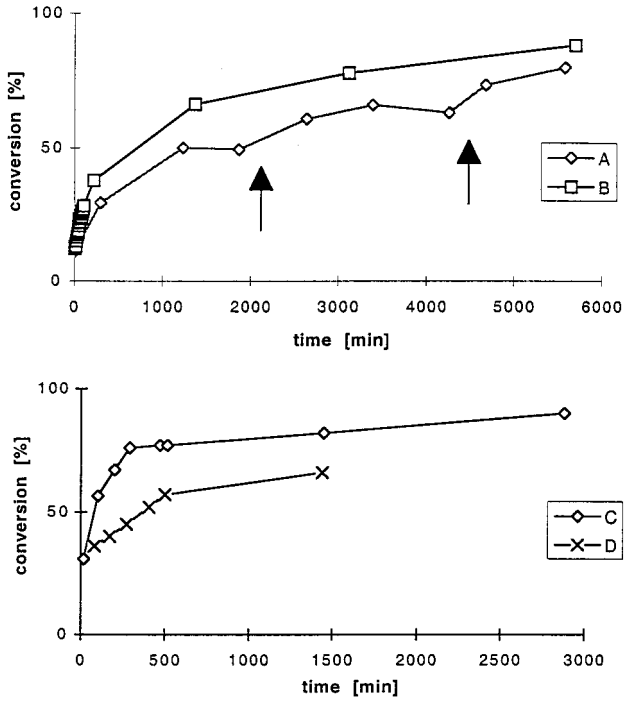

Figure 1. (Above, experiment 1; in situ reaction in the rotor) The formation of **3** as a function of time applying different excesses of LiBr/Et_3N; conversion form **1** to **3** is followed by comparing proton signal H^1 of soluble starting reagent **2** to proton signal H^2 of polymer-linked **3**; (A) 10 equiv of LiBr/Et_3N initially added; arrows indicate the addition of 5 equiv of LiBr/Et_3N portions; (B) 20 equiv of LiBr/Et_3N initially added. (Below, experiment 3) Conversion from **1** to **3** in DMF-h_7 under standard reaction conditions. The proton signal $H^{1''}$, attributed to the carbamate proton of the lysine side chain by 2D Tocsy spectra, is used as reference to quantify the increasing aldehyde signal H^2 of polymer-linked product **3**; (C) represents data from samples taken from the original reaction vessel prior to spectra accumulation; (D) data points are taken from a sample that was kept in the rotor and had no interchange with original reaction conditions.

In a second run we immediately added 20 equiv of Et_3N/LiBr and 5 equiv of dialdehyde **2** to resin in the rotor. As predicted, the reaction was much faster and after around 13 h half of the phosphonodiester was converted to the product (see Figure 1a). By using 30 equiv of Et_3N/LiBr, the reaction half time even further decreased to approximately 7 h (data not shown). In the latter two

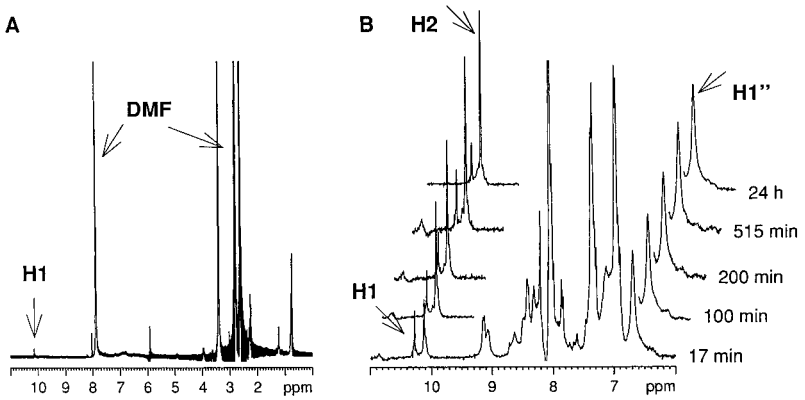

Figure 2. Proton spectra in DMF-h_7 of a resin suspension transferred from a reaction vessel to the NMR rotor at different reaction times applying different pulse sequences: (A) single pulse sequence; (B) LED sequence,[17,18] sine-bell shaped gradients of 12 ms with 32 G/cm for defocusing, and a diffusion delay of 30 ms were used. Without diffusion-encoded measurements, only the signals of DMF and soluble reagents added in excess can be seen. In (B) all protons of **3** can be identified and are clearly distinguished from protons on soluble molecules which are suppressed.

cases, quantitative conversion was attained without the need for adding additional base after prolonged reaction time.

Whereas this experiment clearly demonstrates the feasibility of in situ monitoring of the reaction progress in SPOC, it does not represent a practical way for routine analysis of solid phase reactions. Besides the presently unknown influence of high rotation speeds, the small scale of the reaction in the NMR rotor only approximates realistic reaction conditions. Factors related to higher quantities, the use of gaseous reagents, inert atmosphere, open reaction vessels, and agitation can all have their influence on the successful synthesis. In more realistic conditions with several hundreds of milligrams of resin, where the use of protonated solvent is mandatory, one can sample a few milligrams of resin at different time intervals and prepare the sample for NMR studies through washing, drying, and reswelling in deuterated solvent (experiment 2). To quantify the reaction progress, we added a precise amount of soluble anisaldehyde. Comparison of the integral of the soluble reference (proton $H^{1'}$) and the integral of proton (H^2) of resin-bound product **3** allows quantification of obtained polymer-linked product **3**. In our experiment 86% of the theoretical maximal amount of product was detected, thereby underscoring the success of the nine-step synthesis. For comparison and verification, **4** was cleaved from the resin by adding TFA/5% H_2O, precipitated in ether and isolated by centrifugation. Characterization of the cleaved reaction product **4** indicated a yield of 87.2% and a purity of 91% (determined by HPLC). Whereas these results are in excellent agreement with those of the HR MAS analysis, two factors can potentially interfere with the correct HR MAS quantification of resin bound structures. A first imprecision can come from the theoretical loading of the resin, which may have changed during the synthesis. Premature cleavage of molecules as well as the incomplete removal of solvent and/or moisture can result in incorrect weighing and therefore lead to errors in the evaluation of resin loading. This is a general problem for solid phase synthesis, since the loading (mmol/g) is usually controlled only before or after the immobilization of the first building block. All treatments of the resin thereafter may change this value. The two experiments presented until now suffer from this problem, since the theoretical amount of polymer-linked product **3** is used to quantify the reaction but is calculated with a loading that was determined nine steps before the Horner−Emmons reaction. Second, aggregation of the product molecule on the resin was observed by our group during the synthesis of difficult peptide sequences,[12] leading to very broad NMR signals and preventing a correct integration. However, we never observed this for simple organic reactions, and the broad aspect of NMR lines in the case of aggregation immediately indicates the problem.

The second experiment follows the reaction progress in realistic conditions, but is hampered by time-consuming sample preparation steps required for NMR analysis. This procedure, inconvenient if in situ characterization of fragile intermediates of resin-bound structures directly from the reaction vessel is desired, or if a lot of samples have to be analyzed, as is the case for combinatorial chemistry optimization, can be successfully avoided by exploiting the differential diffusional properties of the soluble part of the sample (solvent and soluble reagents) compared to the resin-attached molecules (reaction products). Our third experiment allows the application of original reaction conditions without any constraints on the sample or on reaction conditions, since the transfer of a resin including all components of the reaction medium (solvent, reagent, catalysts, etc.) from the reaction vessel to the NMR rotor is sufficient to analyze structures bound to solid support with 1D and 2D NMR experiments. The efficiency of our diffusion-filtered measurements is clearly shown in Figure 2, where the ^1H spectra with and without the LED pulse sequence of the reaction mixture (Scheme 1) are shown. Without the diffusion filter, signals of the solvent dominate the spectra. In the spectrum acquired with the LED pulse sequence, however, all disturbing signals are minimized, and the remaining peaks are directly attributed to the immobilized structure on the solid support. After starting the reaction in DMF-h_7 by adding Et_3N/LiBr, we took samples at various times for HR MAS analysis. In a standardized process, the reaction vessel was shaken such as to obtain a homogeneous distribution of resin and solvent. A well-defined amount of this suspension, taken out by means of a pipette, was transferred to the NMR

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rotor, into which a small amount (5 μL) of DMF-d_7 was added to provide the lock signal, and spectra were recorded during 12 min with the LED sequence. As reaction quantification by adding an external standard is not possible in this experiment, since all signals of soluble reagents are suppressed by the LED pulse sequence, the integral of the H^2 proton of the polymer-linked product was compared to the integral of the well distinguishable carbamate proton peak ($H^{1''}$) of the side chain of lysine of polymer-linked **1** and **3**. Although similar to the quantification procedure used in liquid-state NMR, the use of a LED sequence introduces two possible sources of error. Because it stores the magnetization along the *z* axis during the diffusion delay, where spin−lattice (T_1) rather than spin−spin relaxation (T_2) is active, differential T_1 rates for both resin bound protons might possibly influence the peak volumes. However, relaxation studies have shown that the T_1 times of protons on a resin-bound peptide are of similar magnitude as those for the free peptide, [13] and therefore long compared to the 30 ms delay during which diffusion occurs. Differential spin−spin relaxation during the gradient delay can equally introduce an inaccuracy in the measured peak volumes. Protons that are involved in exchange processes, such as amide protons under basic conditions, should therefore be avoided as reference peaks. [14] Moreover, one should favor the use of strong but short gradient pulses to minimize the time spent by the magnetization in the *xy* plane.

Since peptide synthesis was assumed to be quantitative previous to the Horner−Emmons reaction (verified by Kaiser test[15]), the comparison of the two signals allows us to observe the extent of reagent conversion. More generally, a fixed internal NMR reference immobilized on the resin prior to the synthesis could be used as internal reference for quantification and calibration of the spectra and is the subject of ongoing research in our laboratory. In Figure 1b the results of the "NMR sampling" are shown and smooth conversion from **1** to **3** is observed (90% after 48 h). Since the theoretical loading of the resin is not used as reference, the subsequent error (discussed above) is avoided. This may turn out useful especially for resins such as PEGA[16] where reproducible solvent contents and thus reliable determination of the loading are difficult to obtain because exhaustive drying may damage the polymer. After four consecutive spectra (only the result of the first spectra is shown in Figure 1b), the resin suspensions were returned to the reaction vessel. The necessity to renew samples from the reaction vessel and not to retain one representative sample in the rotor is crucial, since original reaction conditions are only respected in the reaction vessel and not in the HR MAS NMR rotor. To illustrate this point, we did keep a first sample in a separate rotor and acquired spectra with the LED sequence every hour. The long-time conversion in this sample is considerably slower (Figure 1b), which we ascribe to the lower concentrations of soluble reagents in the rotor. Another interesting observation was made by the differential reaction rate of a sample after 45 min of NMR spectra accumulation (44% conversion to **3**) compared to that of a sample from the original reaction vessel after the same time of reaction (36% conversion). This clearly points out the ease with which a precise and fast evaluation of an alteration in reaction conditions (in this case, at least the scale of the vessel and the agitation mode are different for both samples) can be obtained by our methodology within minutes.

In the presented example the molecules immobilized on solid support were always fully characterized by HR MAS 2D NMR experiments, but even single characteristic signals (like the aldehyde peak H^2) can be used to monitor the reaction, without complete assignment of all signals, analogously to FT-IR where usually only few signals are assigned.

Conclusion

Three experiments are described that demonstrate how HR MAS NMR can be used to quantitatively monitor SPOC. The kinetics of chemical reactions on solid support can be followed (i) in situ in the NMR rotor itself, (ii) by comparing NMR signals from reconditioned resins with an external standard, or (iii) by the comparison of signals of two immobilized protons. The third method represents a breakthrough in analysis of solid-phase synthesis, since the application of the diffusion filter allows monitoring of solid-phase reactions without any manipulation of the resin except for transferring a resin-suspension to the rotor. The ease to observe ongoing chemical reactions cannot even be performed this simply in classical liquid phase chemistry, since the separation of the attached molecules and soluble reagents and byproducts is not done by separation techniques but by the NMR experiment itself, giving a definite analytical advantage to SPOC over classical chemistry in solution. The potential applications for synthetic chemistry on solid support are numerous and will definitely help in the acceleration of the optimization process of SPOC. We believe that HR MAS NMR methods will enter the solid-phase chemistry laboratory as an indispensable tool, similar to liquid NMR spectroscopy for organic chemistry in homogeneous phase.

Methods and Materials

Unless otherwise noted, all starting materials were obtained from commercial suppliers and used without further purification. *N*,*N*′-Dimethylformamide (DMF) was distilled from CaH_2 and was stored over molecular sieve (4 Å). Triethylamine was used from a freshly opened bottle and was dried by adding molecular sieve (4 Å) 1 h prior to use. LiBr was kept under a N_2 atmosphere and was dried under reduced pressure in a round-bottom flask heated by a hot-air stream. Resins were used after drying for at least 12 h under reduced pressure at room temperature. It should be noted, that polystyrene−polyethylenglycol resin contains a considerable amount of moisture, which can be detected by NMR. Fmoc-E(tBu)-O-Tentagel (0.19 mmol/g) resin was purchased from Rapp Polymer GmbH (Tübingen, Germany). The Horner−Emmons reaction was carried out under N_2 atmosphere, except for the reaction in the rotor. No special care was taken for the exclusion of moisture during transfer of resin from reaction vessels to HR MAS NMR rotors, and the rotors were manually closed by the standard cap. All spectra were recorded at 300.13 MHz on a Bruker DRX300 instrument equipped with a 4 mm $^1H/^{13}C$ double resonance high-resolution MAS probehead optimized for proton resonance. Resin containing rotors were rotated at 4000 Hz. Chemical shifts for ^1H MAS NMR are reported in ppm relative to TMS as internal standard. The

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TMS signal is lost in diffusion experiments. The spectra in these cases are calibrated by comparison to spectra recorded without diffusion delay.

Synthesis of TG-E(tBu)-G-K(Boc)-L-$COCH_2PO(OC_2H_5)_2$ (1). Peptide synthesis was performed on an ABI 431A using standard Fmoc/tBu strategy. The condensation of diethylphosphonoacetic acid was done manually. In respect to the resin charge, 5 equiv of acid were dissolved in NMP (0.5 M solution) and activated by adding 5 equiv of HBTU, 5 equiv of HOBt, and 10 equiv of DIEA. After a 2 min activation time, the solution was poured onto the tetrapeptide-carrying resin and shaken for 3 h. Complete reaction was confirmed by negative ninhydrin test for amines. The resin was washed thoroughly with NMP and DCM and dried under reduced pressure at rt.

Horner−Emmons Reaction in the Rotor. Experiment 1. Resin **1** was loaded into a rotor, and 5 equiv of terephthalaldehyde **2** in DMF-d_7 (0.2 M) was added. The reaction was started by the addition of the appropriate (mentioned in the text) amount of LiBr/Et_3N in DMF-d_7 (0.66 M). To dissolve LiBr in triethylamine and DMF the solution had to be treated by ultrasonic radiation. Spectra were obtained by acquiring 64 transients. Single pulse sequence, with an additional low power (60 dB) presaturation on the polyethylene glycol resonance during the d1 recycle delay, were used. For resonance assignment, a diffusion-filtered Tocsy spectrum was recorded with a mixing time of 100 ms and 64 transients. The diffusion filter in this case allows suppression of the dominant signals of Et_3N and terephthalaldehyde. Resin containing HR MAS NMR rotors were kept at rt without agitation and were transferred to the NMR spectrometer only for spectra acquisition.

Quantification by Adding Internal Standard. Experiment 2. The NMR rotor was loaded with 5.5 mg of resin **3**. A 35 μL amount of DMF-d_7 and 10 μL of a 0.1 M solution of anisaldehyde in DMF-d_7 were added. After at least 10 min incubation time, a ^{1}H spectrum was acquired during 20 min (64 transients with 4k complex points and a time interval of 15 s between each scan).

Horner−Emmons Reaction in a Reaction Vessel. Experiment 3. An amount of 200 mg of **1** (0.036 mmol) was swollen in DMF-h_7 in a bottom − fritted reaction vessel. Terephthalaldehyde (**2**) (51 mg, 0.38 mmol) in 760 μL of DMF-h_7 was added. The reaction was started by the addition of LiBr (165 mg) and triethylamine (263.7 μL) in 3.8 mL of DMF-h_7. To transfer resin from the reaction vessel to the HR MAS NMR rotor, the reaction vessel was shaken vigorously and approximately 2 mL of the obtained homogeneous suspension were taken out by a pipette. During 2 min the suspension was left in the pipet, where the resin concentrates in the tip. Droplets of the suspension were added to a NMR rotor containing 5 μL of DMF-d_7 (the latter used as locksignal). Following this procedure the NMR rotor contained 10−15 mg of resin and approximately 100 μL of DMF. After NMR spectra were taken, the resin suspensions are returned to the reaction vessel. Spectra were obtained in 12 min by acquiring 128 transients applying the LED pulse-sequence. [10,17−19] Sine-bell shaped gradients of 12 ms with 32 G/cm strength and a diffusion delay of 30 ms were used to eliminate the solvent signals. During the recycle delay, a low power presaturation (60 dB) was applied to eliminate the dominating polyethylene glycol resonance.

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