Pergamon

Tetrahedron 56 (2000) 5163–5167

TETRAHEDRON

Impurity Detection in Solid-Phase Organic Chemistry: Scope and Limits of HR MAS NMR

Pierre Rousselot-Pailley,[a] Delphine Maux,[b] Jean-Michel Wieruszeski,[a] Jean-Louis Aubagnac,[b] Jean Martinez[b] and G. Lippens[a,*]

[a]*UMR 8525 CNRS—Institut de Biologie de Lille-Université de Lille II & Institut Pasteur de Lille, 1 rue du Professeur Calmette, BP447, 59021 Lille Cedex, France*

[b]*UMR 5810, Laboratoire des aminoacides, peptides et protéines, associé au CNRS, Université Montpellier II, Place E. Bataillon, 34095 Montpellier Cedex 05, France*

Received 4 January 2000; accepted 22 May 2000

Abstract—We evaluate the possibilities and limits of HR MAS NMR to detect and quantify side products during solid-phase synthesis, using a model system where pyroglutamate and glutamate are coupled in a well defined ratio. Resins swollen in deuterated and protonated solvent are studied. Use of the LED sequence eliminates the peaks due to the use of protonated solvents, but sensitivity is decreased, and differential losses of magnetization might lead to a biased population estimation. However, as all sample workup steps are eliminated, this technique will be helpful in detecting minority species in solid-phase combinatorial chemistry, and its application at the different steps of the reaction might lead to the early detection of otherwise unidentifiable active components.

Introduction

Solid-phase organic chemistry is generally recognized as a powerful and versatile technique for introducing complexity into chemical synthesis.[1] However, its widespread use still suffers from the lack of rapid and accurate analytical techniques that do exist for chemistry in homogeneous phase. Two basic questions have to be addressed: how to monitor a reaction quantitatively, which relates to the question of efficient reaction optimization, and how to detect side products that can contaminate the final desired product? Whereas the classical cleave-and-analysis strategy can answer both questions, its main problem remains the time required for the different sample workup steps. Therefore, a number of alternative approaches have been developed that aim to analyze the products while bound to the matrix.[2] High Resolution Magic Angle Spinning NMR (HR MAS NMR) was introduced towards this goal, and recent work has shown its power and versatility both in identification and quantification of the tethered products.[3] The use of a diffusion filter, as developed in our laboratory, furthermore allows one to work in protonated solvent, thereby removing even the need for sample workup steps of washing, drying and reswelling in deuterated solvent.[4]

The present paper addresses the second question, on the detection and eventual quantification of side products. This aspect has become even more important in combinatorial chemistry where, during optimization steps, one does not optimize the reaction conditions just for two individual reactants, but for two classes of reactants. Because of time constraints, the reaction optimization is in the best of cases performed for only a few members of each class (e.g. one alcohol and one amine), and, implicitly, it is assumed that these conditions will be applicable to the other members of both categories. Detection of eventual side products in such a test reaction is therefore crucial, as it indicates possible problems for the other reactions, and should help to prevent future problems of unidentifiable active compounds.

Using different ratios of Fmoc protected glutamate and pyroglutamate, Enjalbal et al.[5] created an artificial system containing a known amount of impurities, and used Secondary Ion Mass Spectroscopy (SIMS) to simultaneously quantify the two products coupled on a resin. Upon comparison with the quantification as performed by HPLC after cleavage, general agreement between both methods was concluded, demonstrating that Tof-SIMS with its possibility to work on the individual bead level is a most sensitive approach to detect impurities. However, potential problems remain due to the differential degrees of ionization of the species, and also due to the potentially non-homogeneous spatial distribution of the compounds on the bead.

We use here HR MAS NMR to quantify both products on the same system of doubly coupled beads. After identification of the resonance signals of both molecules, both single

Keywords: solid-phase synthesis; combinatorial chemistry; HR MAS NMR; impurity detection.

* Corresponding author. Fax: +33-03-20-87-12-33; e-mail: guy.lippens@pasteur-lille.fr

0040–4020/00/$ - see front matter
PII: S0040-4020(00)00430-0

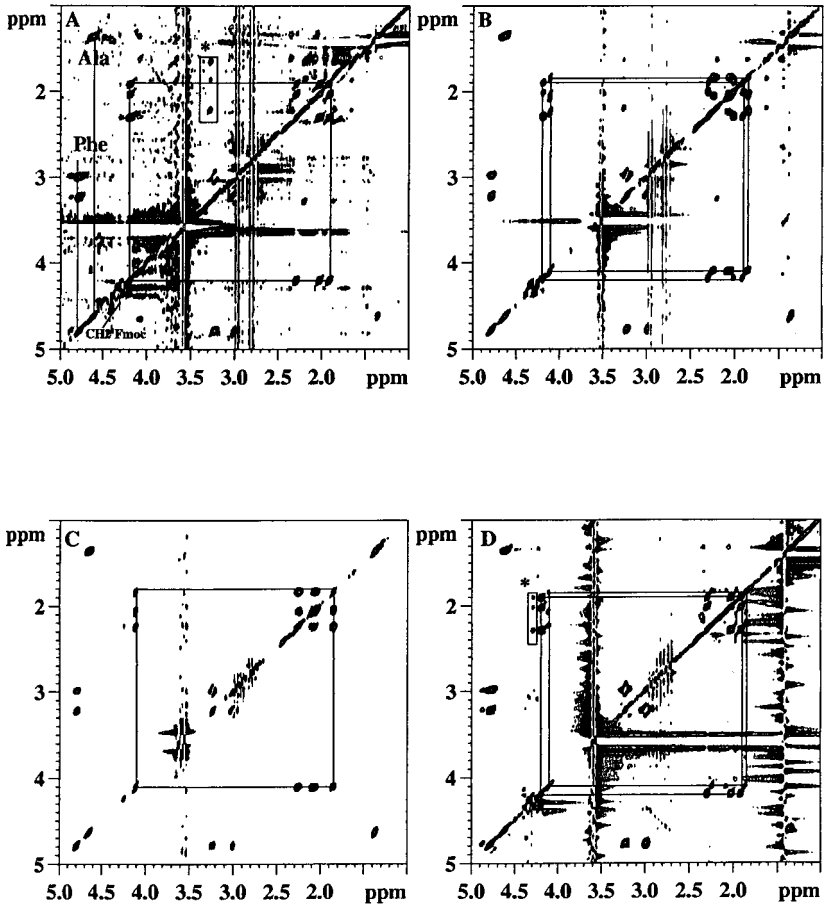

Figure 1. HR MAS TOCSY spectra on 3 mg of: (A) 100% Glu; (B) equimolar quantities of Glu and Pyrglu; (C) 100% Pyrglu containing resins; and on (D) 10 mg of a 1% Pyrglu/99% Glu charged resin. The first three experiments were recorded with 32 scans per increment, whereas the spectrum in (D) was recorded with 72 scans. The boxed region indicated by * in (A) represents the spin system of the deprotected Glu side chains, whereas in (D), we tentatively ascribe these signals to the molecules on the surface of the beads.

pulse spectroscopy and the diffusion filtered experiment are used to quantify both products on the resin, and this under conditions of deuterated and protonated solvent. Whereas impurities of 10% could be easily detected on a macroscopic sample of 3 mg under both solvent conditions, working with 10 mg of resin in deuterated solvent allowed the detection of as low as 1% of impurity.

Results

The ^{1}H assignment could in a straightforward manner be obtained from the MAS TOCSY spectra on the 100% Fmoc protected glutamate (Glu) sample (Fig. 1A), and on the 100% pyroglutamate (Pyr) (Fig. 1C). The TOCSY spectrum of the resin containing both molecules in equimolar quantity confirmed the distinction between the Hα resonances of Glu and Pyrglu (Fig. 1B). Resonance distinction is essential to be able to quantify both molecules, and is in our experience a more stringent requirement on the field strength used than is sensitivity (see below).

Because the Glu Hα proton resonates at almost the same position as one of the Fmoc CH_2 protons, the Pyrglu Hα was chosen as the better candidate for integration (Fig. 2). Assigning a value of 100% to the Phe or Ala Hα peak, integration of this Pyrglu Hα yielded directly a percentage of this compound on the resin.

On each sample in DMF-d7, we first performed a single pulse experiment followed immediately by a second experiment using the Longitudinal Eddy current Delay (LED) pulse sequence.[6] This sequence, recently applied in the framework of HR MAS NMR to eliminate signals of freely diffusing species including protonated solvent,[4] was first used on the sample swollen in DMF-d7, in order to assess possible factors that might influence the quantitative integration. In a second phase, we recorded the spectra with the LED sequence on the same resins swollen in protonated DMF.

The single pulse experiment on the DMF-d7 swollen resins yielded values for the Pyrglu content that were in good agreement with the values previously established by LC-UV after cleavage (Table 1). With 3 mg of resin swollen in DMF-d7 and a total of 64 scans resulting in a 4 min measuring time, where a sufficiently long recycling delay (3 s) was used to ensure complete magnetization relaxation, impurities as low as 5% can be easily detected in a quantitative manner. Of course, at lower field strengths, this limit will require a longer acquisition time, but even a 3 fold increase in measuring time to compensate for the lower

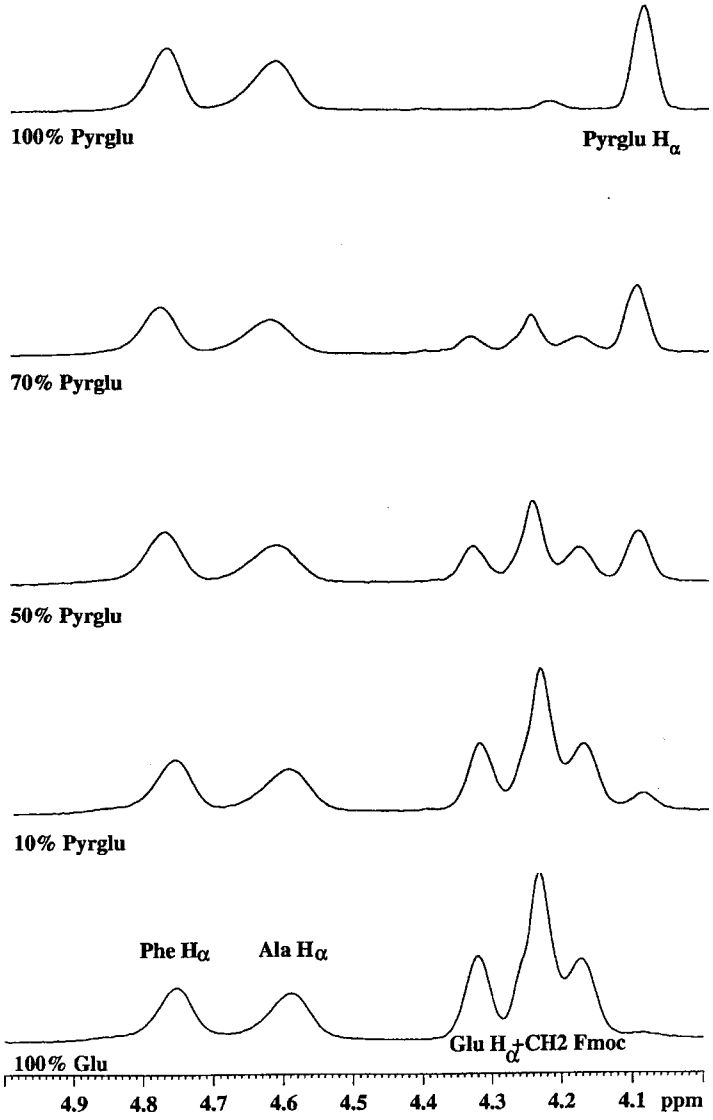

Figure 2. 1D spectra of the different resins swollen in DMF-d7 acquired with a single pulse experiment. The signals of the CH_2 protons of the Fmoc protecting group and the Glu Hα cannot be easily distinguished.

field strength on a 300 MHz spectrometer still leads to an acceptable experiment time of 12 min.

To further evaluate the sensitivity of the method, we prepared a resin with a theoretical loading of 1% Pyrglu. After cleavage, LC-UV was unable to detect any Pyrglu, but a HR MAS NMR experiment on 10 mg of resin over a similar time interval yielded a value of 2%. This slightly higher percentage than the theoretical value observed not only for the 1% resin but also for the other charges could

Table 1. Percentages of Pyrglu as determined by the content of this component in the coupling mixture (column 1), LC-UV after cleavage (column 2), a single pulse experiment on 3 mg of sample in deuterated DMF (column 3), a LED sequence on the samples (columns 4 and 5) and, finally, the samples swollen in protonated DMF (columns 6 and 7)

Theoretical percentage of Pyrglu (%)	Percentage LC-UV (%)	DMF-d7			DMF-H7	
		Single pulse (%)	Diffusion without correction (%)	Diffusion with correction (%)	Diffusion without correction (%)	Diffusion with correction (%)
0	0		0	0	0	0
1		2				
5		5				
10	14	19	20	16	22	19
50	56	54	61	49	64	49
70	75	76	83	66	83	64
100	100	100	125	100	130	100

point to a more efficient coupling of Pyrglu. A possible reason for this is the slower diffusion into the resin of the more bulky protected glutamate.

In order to probe the limits of the HR MAS NMR technique, we recorded an overnight TOCSY experiment on the 10 mg of 1% Pyrglu loaded resin. To our surprise, we saw not only the spin connectivity from the Pyrglu Hα to its side chain, but also a second minor form next to the major Glu side chain (Fig. 1D). The same resonance line was seen with comparable intensity in the 50% Glu/50% Pyrglu sample, excluding a nearest neighbor effect of the Pyrglu molecules on the proximate Glu side chains. As a similar minor form was observed for the Ala and Phe Hα resonances, we tentatively assigned these signals to the fraction of peptide chains that are bound to the surface of the beads, and that therefore experience a slightly different magnetic environment than the ones bound in the interior of the bead. Upon use of deuterated benzene as solvent, this signal disappeared. Integration of the cross peaks in DMF-d7 yielded a population of 3%, of the same order of magnitude as the estimated surface charge.[7] In the 100% A sample, we moreover detected a small residual Glu line at 3.2 ppm (Fig. 1A*), corresponding to a small amount of N-terminal deprotected peptide chains due to DMF degradation in the rotor. This again demonstrates the analytical power of the technique not only to detect but also to identify residual signals on the resin.

Immediately following the single pulse experiment, we recorded a diffusion filtered spectrum of the same DMF-d7 swollen resins. As noted previously, this experiment is less sensitive because of magnetization losses due to relaxation (T_2 during the gradient periods, and T_1 during the diffusion delay), to J coupling evolution during the gradient delay and, finally, to imperfections in the five pulses of the LED sequence. An additional problem is that these factors are not necessarily constant for both molecules, and might therefore bias the quantification procedure in a similar way as differential degrees of ionization would do to a quantification by the Tof-SIMS technique. One factor that can differentially affect two resonances is the difference in relaxation times, where it is known that the last coupled residue has longer relaxation times than the other residues.[8]

The resin containing exclusively one of the molecules allowed us to experimentally determine this correction factor as the ratio of the integral of the Pyrglu Hα proton line in the LED spectrum to the average of the Phe and Ala Hα proton integrals. After correction, the diffusion experiments indicated a Pyrglu content slightly less than the percentages as determined by LC-UV analysis, which we took as the reference value, and also smaller than the values as determined by the single pulse sequence. Still, deviations for the three methods and for all samples were less than 10%. Finally, we took spectra on the same samples in DMF-h7 with the LED sequence, and found good agreement after correction with the previously determined values (Table 1).

Conclusion

We have evaluated the possibilities and limits of HR MAS NMR to detect and quantify side products during solid phase synthesis. If well separated signals are available for the different molecules, reliable quantification and identification of impurities present in concentrations as low as 1% can be obtained when using a single pulse experiment on resins swollen in deuterated solvent. The LED sequence allows the use of protonated solvents, but sensitivity is decreased, and differential losses of magnetization can lead to a biased population estimation. Still, as all sample workup steps are eliminated, this technique will be helpful in detecting minority species in solid phase combinatorial chemistry, and its application at the different steps of the reaction might lead to the early detection of otherwise unidentifiable active components.

Experimental

The resin samples with attached peptides were identical to the ones used for the TOF-SIMS evaluation.[5]

MAS NMR experiments

After loading of 3 mg of one resin in the 4 mm rotor DMF-d7 was added to swell the resin. Tetramethysilane (TMS) was added as a internal reference to the solvent before the resin swelling. All NMR experiments were performed at 298 K on a BRUKER DMX 600 MHz spectrometer (Bruker Spectrospin, Germany) equipped with a 4 mm HR MAS probe using a 6 kHz spinning rate. TOCSY spectra were recorded on 3 mg of resin with a 60 ms MLEV-17 mixing time with 32 scans per increment for most spectra, except for the 1% Pyrglu/99% Glu resin where 10 mg of resin and 72 scans were used. Further acquisition parameters were: 400 complex points in the t_1 dimension and 1024 complex points in t_1. Gradient parameters for the LED based sequence were as previously described.[4] Spectral processing and integration were performed with XWINNMR from BRUKER.

Acknowledgements

The NMR facility used in this study was funded by the European Community (ERDF), the Région Nord—Pas de Calais (France), the CNRS and the Institut Pasteur de Lille. P. R.-P. and D. M. are funded by a doctoral fellowship of the Ministry of Research and Technology (MRT; France).

References

1. Hermkens, P. H. H.; Ottenheijm, H.C.J.; Rees, D. C. *Tetrahedron* **1996**, *52*, 4527–4554; Hermkens, P. H. H.; Ottenheijm, H. C. J.; Rees, D. C. *Tetrahedron* **1997**, *53*, 5643–5678; Obrecht, D.; Villalgordo, J. M. *Solid-Supported Combinatorial and Paralles Synthesis of Small-Molecular-Weight Compound Libraries*; Elsevier: Oxford, 1998.

2. Cilli, E. M.; Marchetto, R.; Schreier, S.; Nakaie, C. R. *Tetrahedron Lett.* **1997**, *38*, 517–520; Johnson, C. R.; Zhang, B. *Tetrahedron Lett.* **1995**, *36*, 9253–9256; Look, G. C.; Holmes, C. P.; Chinn, J. P.; Gallop, M. A. *J. Org. Chem.* **1994**, *59*, 7588–7590; Russel, K.; Cole, D. C.; McLaren, F. M.; Pivonka, D. E.

J. Am. Chem. Soc. **1996**, *118*, 7941–7945; Yan, B.; Jewell, C. F.; Myers, S. W.*Tetrahedron* **1998**, *54*, 11755–11766; Yan, B. *Acc. Chem. Res.* **1998**, *31*, 621–630.
3. Anderson, R. C.; Stokes, J. P.; Shapiro, M. J. *Tetrahedron Lett.* **1995**, *36*, 5311–5314; Anderson, R. C.; Jarema, M. A.; Shapiro, M. J.; Stokes, J. P.; Zilix, M. J. *J. Org. Chem.* **1995**, *60*, 2650–2651; Fitch, W. L.; Detre, G.; Holmes, C. P.; Shoolery, J. N.; Keifer, P. A. *J. Org. Chem.* **1994**, *59*, 7955–7956; Keifer, P. A. *J. Org. Chem.* **1996**, *61*, 1558–1559; Keifer, P. A.; Baltusis, L.; Rice, D. M.; Tymiak, A. A.; Shoolery, J. N. *J. Magn. Reson. A.* **1996**, *119*, 65–75; Sarkar, S. K.; Ggarigipati, R. S.; Adams, J. L.; Keifer, P. A. *J. Am. Chem. Soc.* **1996**, *118*, 2305–2306; Pop, I.; Dhalluin, C.; Depréz, B.; Melnyk, P.; Lippens, G.; Tartar, A. *Tetrahedron* **1996**, *52*, 12209–12222.
4. Warrass, R.; Wieruszeski, J. M.; Lippens, G. *J. Am. Chem. Soc.* **1999**, *121*, 3787–3788.
5. Enjalbal, C.; Maux, D.; Subra, G.; Martinez, J.; Combarieu, R.; Aubagnac, J. L. *Tetrahedron Lett.* **1999**, *40*, 6217–6220.
6. Altieri, A. S.; Hinton, D. P.; Byrd, R. A. *J. Am. Chem. Soc.* **1995**, *117*, 7566–7567; Gibbs, S. J.; Johnson, C. S., Jr. *J. Magn. Reson.* **1991**, *93*, 395–402; Dinggley, A. J.; Mackay, J. P.; Chapman, B. E.; Morris, M. B.; Kuchel, P. W.; Hambly, B. D.; King, G. F. *J. Biomol. NMR* **1995**, *6*, 321–328.
7. Hodge, P. *Synthesis and Separation using Functional Polymers*; Sherrington, D. C., Hodge, P., Eds.; Wiley: New York, 1988, pp 43–122.
8. Elbayed, K.; Bourdonneau, M.; Furrer, J.; Richert, T.; Raya, J.; Hirschinger, J.; Piotto, M. *J. Magn. Reson.* **1999**, *136*, 127–129.