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Monitoring the allyl ester deprotection by HR MAS NMR in BAL-solid phase peptide synthesis

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The backbone amide linker strategy, in which the growing peptide chain is anchored to a solid support via a backbone amide nitrogen, has proven to be successful for the synthesis of cyclic peptides. Optimisation of the reaction conditions for the synthesis of c(Gly-Trp-βAla-Phe) could be accomplished by the help of high resolution magic angle spinning (HR MAS) NMR and the results are presented here. Signal vanishing of HR MAS NMR resonances were encountered and proven to be originated from interchain aggregations of peptide chains.

Keywords: backbone amide linker; HR MAS NMR; solid phase; rotational mobility; allyl deprotection; signal vanishing; interchain aggregations

Introduction

Since the introduction of the concept of solid phase peptide synthesis (SPPS) by Bruce Merrifield in 1963 [1], this synthetic strategy has evolved into a very efficient tool for the synthesis of peptides and even small proteins. The breakthrough of combinatorial chemistry has increased the needs for solid-phase synthesis, even outside the domain of peptides, e.g. for the synthesis of small organic molecules [2].

Besides the many advantages of this technique that pushes the reaction to completion using an excess of reagents and the subsequent easy separation of non-reacted reagents by filtration, the lack of good analytical techniques for resin-bounded compounds had soon become a major limitation since the classical method of 'cleave and analyse' can be very time consuming and also alter the actual characteristics of the grafted species [3].

Very few methods allow direct and non-destructive on-resin analysis of the growing peptide chain. 'On-bead' FT-IR [4] is a well suited method to monitor conversions of functional groups. Its use is therefore restricted to specific reactions. ^{13}C gel-phase-NMR – where standard NMR equipment can be used – has proven to be a reliable technique to determine the success or failure of a chemical transformation. However, due to the low sensitivity inherently linked to the 1% natural abundance of ^{13}C and to the small amount of compound attached to the resin, spectrum acquisition is very time consuming [5,6]. In the last decade, high resolution magic angle spinning (HR MAS) NMR has become a commonly used analytical method for resin-bound molecules [7].

The magic angle spinning (MAS) technique, which cancels magnetic field heterogeneity at interfaces of heterogeneous samples, combined with the use of appropriate solvents swelling up of the supporting matrix, results in vanishing dipolar interaction thanks to local rotational mobility, making the use of classical liquid NMR techniques amenable to the analysis of resin-bound compounds. Recent improvements of the technique, such as the application of diffusion filters [7,8] and specific pulse sequences [7] [f.i. Carr-Purcell-Meiboom-Gill (CPMG) spin echoes] have made of HR MAS NMR a very powerful tool for the characterisation and, with the needed care, quantification of compounds grafted to their solid support [8,9].

It was demonstrated that peptides and intermediates could be fully characterised while still bound to the resin [10,11]. HR MAS NMR also showed that a regular helical structure and other secondary structures can be adopted by a resin-bound bioactive peptide [6,12]. Dhalluin *et al*. [6] showed that incomplete coupling steps in the synthesis of commonly called 'difficult sequences' in SPPS were due to aggregations of peptide chains at a critical length. A correlation was made between poor synthetic results, aggregation of peptide chains at the interface, and line broadening or even signal vanishing in HR MAS NMR spectra [13]. Influences of different types of resin [7,14] and solvents [7] on the resolution of HR MAS NMR spectra have also been investigated.

One of our research programmes required the synthesis of cyclic tetra- and pentapeptides. We decided to use the backbone amide linker (BAL) approach, in which the growing peptide chain is anchored to a solid support via a backbone amide nitrogen, which, after suitable deprotection steps, leaves the *N*-terminal amine and the *C*-terminal carboxyl group available for cyclisation on the resin [2,15].

Here, we report the application of HR MAS NMR as a tool for optimising the experimental conditions for the critical step in the solid-supported synthesis of these cyclic peptides: the allyl ester deprotection. Three types of aminomethylated resins were investigated: HypoGel, ChemMatrix and Merrifield resin. (Figure 1) Failure of the deprotection reaction is clearly indicated by the presence of undesired allyl signals, whereas upon successful deprotection, all signals of the grafted peptide disappeared, which could be attributed to a loss of rotational mobility of the peptide.

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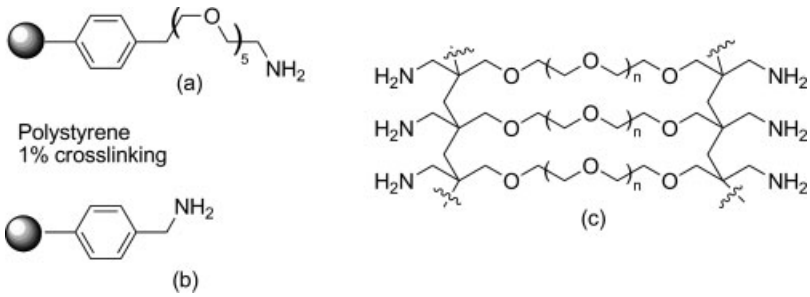

Figure 1. Structures of aminomethyl HypoGel-NH_2 200 (a), aminomethyl Merrifield resin (b) and aminomethyl ChemMatrix (c).

Materials and Methods

General Methods and Reagents

Boc- or Fmoc-protected amino acids were obtained from NovaBiochem or Fluka. Aminomethyl polystyrene was purchased from NovaBiochem, ChemMatrix from Matrix Innovation and aminomethyl HypoGel from RAPP Polymere. H-Phe-OAll.HCl was provided by NeoSystem and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) by Senn Chemicals. Other reagents for peptide synthesis and solvents were obtained from Sigma-Aldrich and Acros. Liquid hydrogen fluoride (HF_{liq}) was purchased from Praxair. The synthesis was typically performed on 0.5 g of resin.

All isolated analogues were analysed using a Waters Breeze analytical HPLC on a reversed phase C18 column (Discovery BIO SUPELCO Wide Pore C18 column, 25 cm × 4.6 mm, 5 µm) with a linear gradient (3–100% CH_3CN in H_2O, containing 0.1% TFA, in 20 min.) at a flow rate of 1 ml/min. with UV detection at 215 nm. Mass spectra were recorded on a VG Quatro II spectrometer (electrospray ionisation, cone voltage 70 V; Micromass, Manchester, UK) with the MassLynx 2.22 software (Micromass) for data analysis.

Proton HR MAS NMR Spectra

All high resolution magic angle spinning (HR MAS) NMR spectra were acquired from samples containing $CDCl_3$ using zirconium oxide rotors (4 mm), on an Avance2 Bruker 500 spectrometer using a dedicated HR MAS triple channel probe tuned to the resonance frequencies of ^1H (500.13 MHz), ^{13}C (125.77 MHz) and ^{119}Sn (186.50 MHz) nuclei, equipped with a z-gradient coil reaching a maximum of 53.5 G/cm of field gradient intensity. Only the proton channel was used in this work. The amount of resin used was 15–20 mg in 120 µl rotors. Unless indicated otherwise, the HRMAS spectra presented were obtained in the standard acquisition mode or in CPMG spin echo mode, using standard pulse programmes from Bruker program library. HR MAS spectra acquired in the diffusion-filtered mode, aiming at filtering out all NMR signals arising from non-grafted species (non-deuterated solvent molecules, excess liquid state reagents, impurities, side-products) were obtained by the longitudinal eddy current delay (LED) diffusion-filtered pulse sequence, likewise available in the standard Bruker pulse programme library as 'ledgp2s1d', with 70% of the maximum gradient intensity, using SIN.100 gradient pulse. The number of scans was typically 64, a relaxation delay D1 = 3 s, the diffusion delay was 30 ms, and the gradient recovery delay 3 ms.

Synthesis of Cyclic Peptides on Resin using a BAL strategy

Coupling of the BAL-linker 5-(4-formylphenoxy)pentanoic acid [16] to the resin was achieved using a three molar excess of a solution of equimolar quantities of the BAL linker, 1-hydroxybenzotriazole (HOBt) and *N*,*N*′-diisopropyl carbodiimide (DIC) in dry DMF after shaking during 24 h at room temperature. The first amino acid, introduced as an allyl ester (Scheme 1) was coupled via reductive amination using 4 equivalents of H-AA-OAll and 4 equivalents of $NaCNBH_3$ in a mixture of DMF : MeOH (8/2). This mixture was added to the resin for 18 h. For the subsequent coupling to the secondary amine, the symmetrical anhydride coupling was used: 5 equivalents of Boc-AA-OH or Fmoc-AA-OH were dissolved in a mixture of DCM : DMF (9 : 1) and cooled to 0 °C. 2.5 equivalents of DIC were added and the solution was stirred for 30 min at room temperature. The resulting symmetrical anhydride was then added to the resin for 24 h. This procedure was applied twice [2,15,17].

Peptide chain elongation was performed with the classical Boc- or Fmoc-strategy using a threefold excess of N_α-protected amino acid, HOBt, and DIC in DMF/DCM (1/1). Removal of the N_α-protecting group was achieved by stirring the resin in a solution of TFA/CH_2Cl_2/anisole (49/49/2) (Boc-deprotection) or in a mixture of 20% piperidine in DMF (Fmoc-deprotection) during 5 and 15 min. All these reactions were followed using colour tests: the Kaiser test [18] was used to monitor the coupling of the BAL linker to the resin, the side-chain elongation as well as the cyclisation. The reductive amination was followed using the DNPH test [19], and the NF31 test [20] was used to confirm the coupling of the second amino acid to the secondary amine. Allyl deprotection was first carried out using an excess of $PhSiH_3$ (24 equivalents) and a catalytical amount of $Pd(PPh_3)_4$ (0.2 equivalents) in dry DCM during twice 15 min [21]. Better results were obtained when the reaction time was prolonged to 3 h, without any repetition (*cf* Results and Discussion). After final Boc- or Fmoc-deprotection, the peptide was cyclised on resin using 3 equivalents of TBTU and 6 equivalents of DIPEA in a DMF : DCM mixture, for 3 h.

The so-formed cyclic peptide was cleaved off from the resin by the treatment with liquid hydrogen fluoride at 0 °C for 90 min, in the presence of *p*-cresol and *p*-thiocresol for compound **5** and in the presence of anisole for compounds **12** and **13**. After removal of the HF under reduced pressure, the crude peptide was precipitated in anhydrous ether, filtered, dissolved in acetic acid and lyophilised.

Results and Discussion

The so-called backbone amide linker (BAL) approach, in which the growing peptide chain is anchored to a solid support via a backbone amide nitrogen instead of via its carboxyl group, allows

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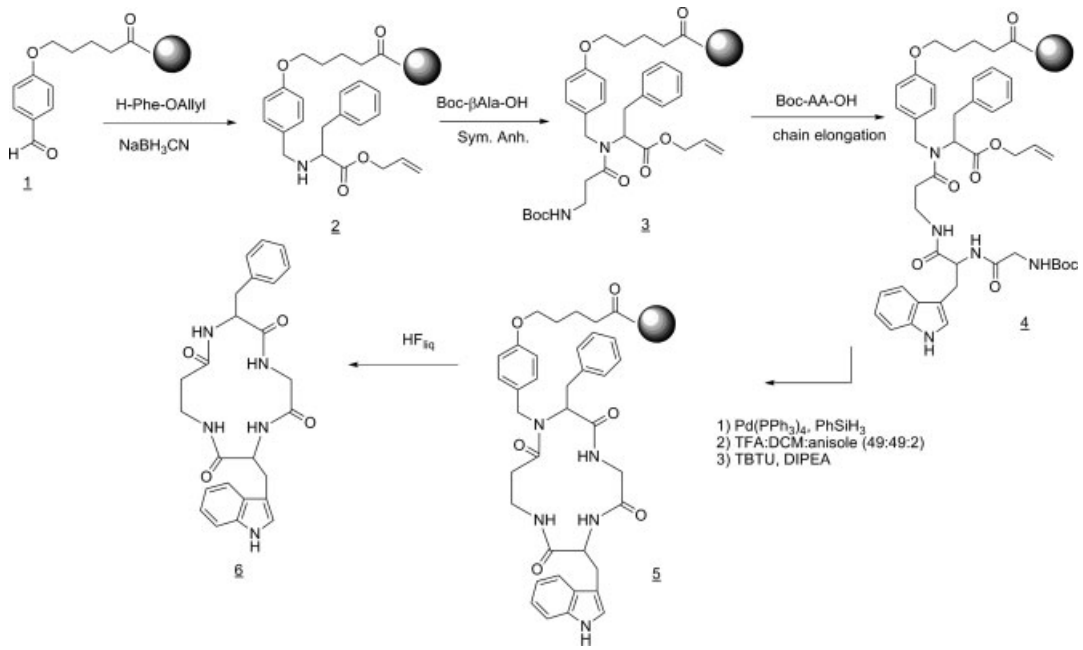

Scheme 1. General scheme for the synthesis of c(Gly-Trp-βAla-Phe) via the BAL strategy.

to overcome the limitations linked to *C*-terminal anchoring or side chain anchoring and gives access to *C*-terminal modified and cyclic peptides [2,15]. The cyclisation reaction can be performed prior to the release of the deprotected cyclic peptide from the resin. The conditions for the release of the cyclic peptide can be fine tuned by the proper choice of the linker [17]. We decided to use the 5-(4-formylphenoxy)pentanoic acid linker, which is resistant to TFA treatment and thus allows Boc- as well as Fmoc-chemistry, and also easily allows the removal of side-chain protective groups. For the cyclisation reaction, the *C*-terminal protective group has to be removed selectively. The allyl ester is most commonly used for this purpose, because it can be removed by the treatment with a Pd^0 catalyst in the presence of a nucleophile-like phenylsilane [21] (Scheme 1). Whereas all coupling reactions, including the cyclisation, can be monitored using colour tests such as the Kaiser test, the quantitative removal of the allyl ester cannot be determined by a colour test. We therefore used HR MAS NMR to determine the best conditions.

Synthesis and HR MAS NMR Analysis of c(Gly-Trp-βAla-Phe)

Cyclic tetrapeptides containing one beta-homo amino acid are well known β-turn scaffolds [22]. In order to obtain high resolution of HR MAS NMR signals, the ligand that is grafted to the solid support needs to display a high degree of isotropic conformational mobility. Therefore, aminomethyl HypoGel resin (HypoGel-NH_2 200, loading 0.9 mmol g^{-1}) (Figure 1) was our first choice for the synthesis of a model peptide, c(Gly-Trp-βAla-Phe). This type of resin was chosen because it ensures the required conformational mobility owing to its five ethylene glycol units, which give a very characteristic peak at 3.7 ppm in the HR MAS ^{1}H NMR spectrum. The 5-(4-formylphenoxy)pentanoic acid linker was coupled to this solid support, and all reactions were monitored using colour tests and HR MAS NMR analysis.

After the reductive amination reaction with Phe-OAll, the typical aldehyde proton of the linker around 10 ppm vanished, whereas the allyl proton resonances appear in the range 4.5–4.6 ppm (broad non-first-order pattern) and at 5.23, 5.55 and 5.85 ppm. These chemical shifts are in good agreement with values that can be obtained from data tables and/or empirical, increment-based calculations. [23] To demonstrate the ability of HR MAS NMR to detect the removal of the *O*-allyl ester, Boc-Gly was coupled to the secondary amine using the symmetrical anhydride method [2,15,17]. It has been well documented [2,15] that after *N*-deprotection, the resulting backbone-linked dipeptide has a high tendency to cyclise to the diketopiperazine. This is clearly demonstrated by the disappearance of the allyl proton resonances in the spectrum of a sample that was neutralised after Boc-deprotection, and allowed to react for 3 h (Figure 2). The formation of the diketopiperazine can be avoided by using *in situ* neutralisation during coupling [2,15], thus allowing further peptide synthesis. Also, when Boc-βAla is used, as required for our target peptide, cyclisation to form a diazepinedione is not observed. Further peptide synthesis using DIC/HOBt couplings and Boc deprotections using 50% TFA could be monitored by the Kaiser test, and the HR MAS spectra showed the typical appearance and disappearance of the characteristic Boc signal at 1.4 ppm (Figure 3). The standard conditions for *O*-allyl removal, using a catalytic amount of $Pd(PPh_3)_4$ (0.2 equivalents) and an excess of $PhSiH_3$ (24 equivalents) in dry DCM during twice 15 min were applied [21].

The successful deprotection was proven by the disappearance of the allyl signals in the spectrum. (Figure 3f). Subsequent Boc-deprotection and cyclisation using TBTU activation during 3 h resulted in a negative Kaiser test, and the peptide-resin was treated with liquid HF in the presence of p-cresol and p-thiocresol for 90 min at 0 °C. However, no pure cyclic peptide could be isolated, even though LC/MS analysis shows the presence of the m/e peak (462.3855) of the target product, contaminated however, with numerous other m/e values, that correspond to the target m/e, increased by *n* times the m/e increment of 44 g mol^{-1}. These are associated with ethylene glycol units resulting from PEG-spacer decomposition (Figure 4). This indicates that the HypoGel linker is also cleaved during reaction in HF_{liq}.

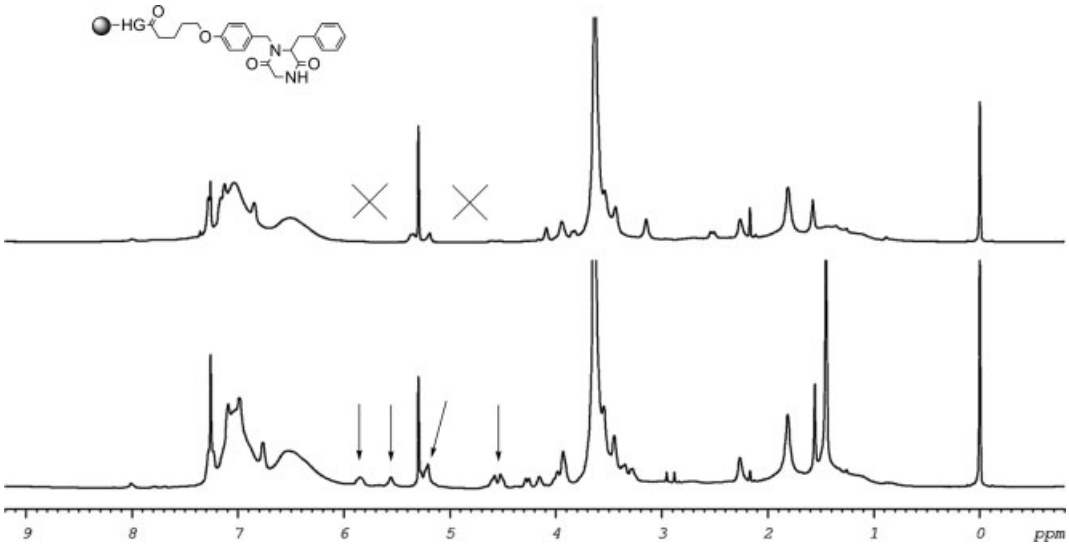

Figure 2. HR MAS NMR spectrum in $CDCl_3$ before (bottom) and after Boc-deprotection of Gly: the disappearance of the allyl signals indicates DKP formation (top).

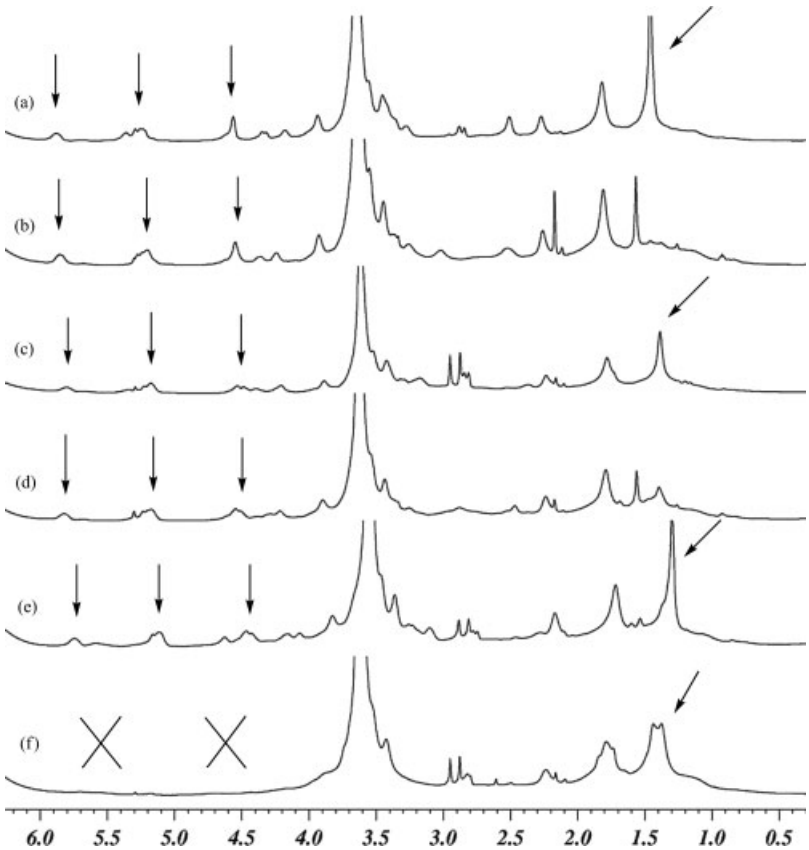

Figure 3. Expansion of the HR MAS NMR spectra displaying the monitoring of the amino acid coupling and allyl deprotection in the synthesis of c(Gly-Trp-βAla-Phe) (Scheme 1). (a) Coupling of Boc-βAla, **3** (b) **3** after TFA treatment, (c) after coupling of Boc-Trp, (d) after Boc-deprotection, (e) coupling of Boc-Gly, **4** (f) **4** after allyl removal, before Boc-deprotection. The arrows indicate the Boc and allyl signals.

ChemMatrix, which is composed exclusively of primary ether bonds with a highly cross-linked matrix (Figure 1), is reported to be highly chemically stable and resistant to liquid HF treatment [24]. We therefore synthesised the model peptide on this resin (loading 1.3 mmol g^{-1}), using the same synthesis protocol. After construction of the linear peptide and cleavage from the resin using HF_{liq}, the linear peptide was shown to be 90% pure according to LC/MS analysis.

Monitoring the allyl deprotection under the conditions described for the HypoGel resin, by HR MAS NMR showed the disappearance of the allyl signals. After Boc-deprotection and cyclisation with 3 equivalents of TBTU and 6 equivalents of DIPEA for

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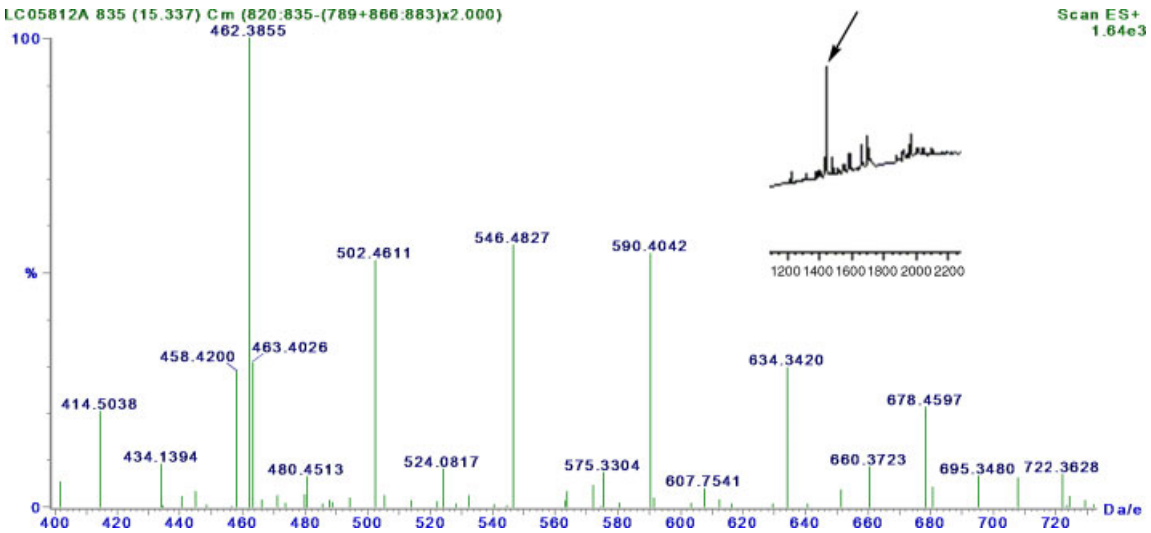


Figure 4. Mass spectrum of the major peak in the LC chromatogram showing the presence of the correct mass (M + 1 = 462 g mol^{-1}) and of masses of fragments differing by 44 g mol^{-1} increments. Insert: section of the HPLC chromatogram indicating the major peak. This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci.

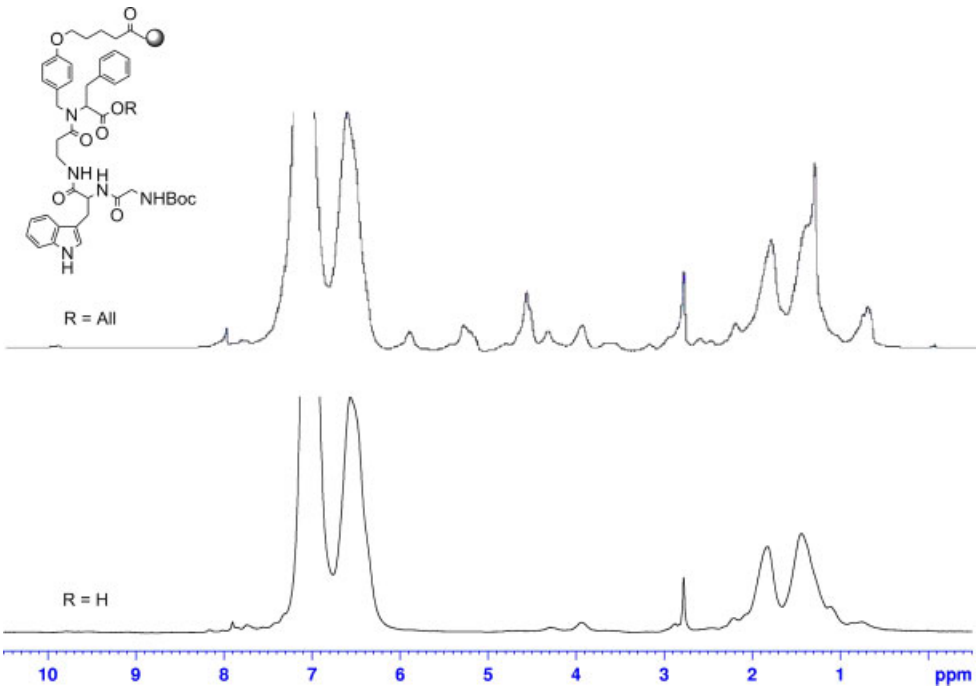


Figure 5. Disappearing of all signals after allyl ester deprotection (bottom). Only signals rising from resonances of the solid support are still visible but also reduced. This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci.

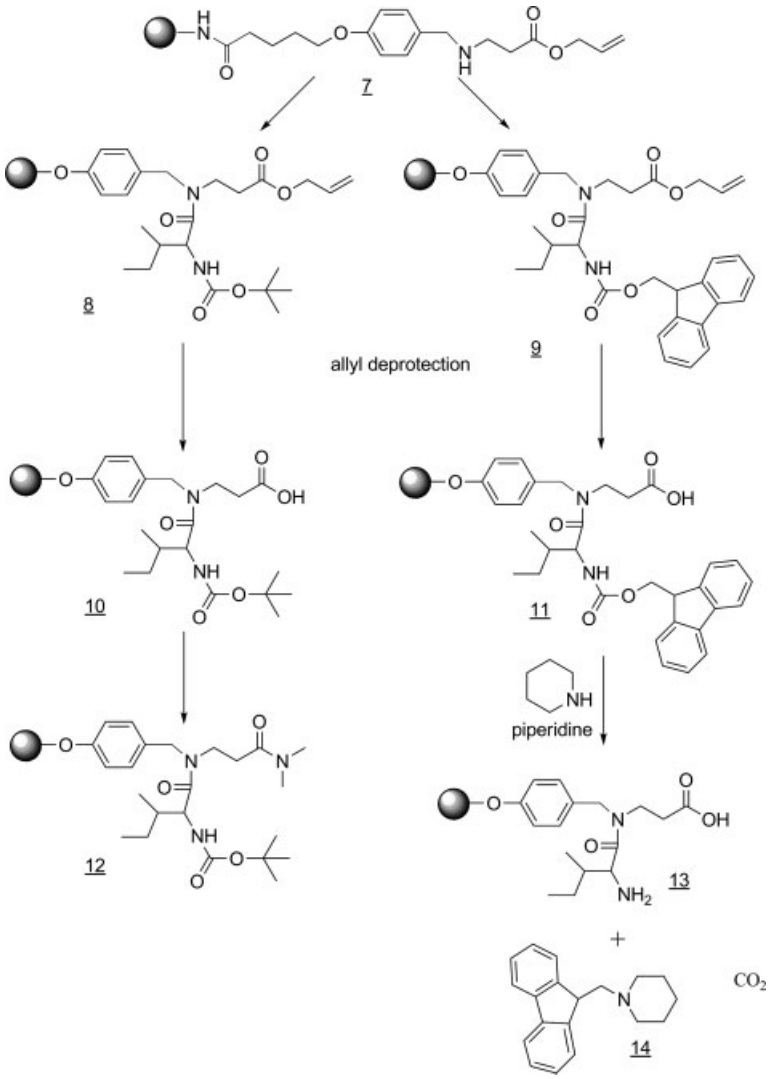
4 h (reaction followed by the Kaiser test), the cyclic peptide could be cleaved from the resin. LC/MS analysis shows that the major peak in the HPLC chromatogram has a molecular mass corresponding to the correct peptide. No indications of decomposition of the resin were observed. However, in subsequent syntheses, the applied procedure of twice 15 min for the cleavage of the allyl ester did not show the required reproducibility, and the HR MAS NMR spectrum did not show the expected spectral pattern of the target graft. As a consequence, we analysed all filtrates by LC/MS to figure out whether the peptide was split off, but no peptide was ever found, only excess reagents were visible. This proves that no product was cleaved off from the resin, thus that the peptide was presumably still anchored to the solid support, without any evidence. In addition, the HR MAS spectra showed that the allyl deprotection was not always reproducible when the latter ChemMatrix support was used. Therefore, in order to find out why the graft signals vanish, the synthesis of the model peptide c(Gly-Trp-βAla-Phe) was attempted on aminomethylated Merrifield resin (loading 0.45 mmol g^{-1}), since this procedure is known from literature to be successful.

The linear peptide sequence was assembled using the same procedures and the coupling reactions were followed with the Kaiser test and HR MAS NMR. However after treating the solid-supported peptide twice with the palladium catalyst and

phenylsilane for 15 min, the allyl signals were still visible in the HR MAS spectrum, indicating that the allyl deprotection was at least not complete. Prolongation of the reaction to three times 15 min or once 2 h and the use of freshly prepared $Pd(PPh_3)_4$ as well as other allyl deprotection protocols mentioned in the literature, such as the use of 3 equivalents catalyst in $CHCl_3$: AcOH : NMM (37 : 2 : 1) for 2 h [25], were tried, but without success.

Since this allyl removal went smoothly on the HypoGel resin, which contains a long penta ethylene oxide spacer, but an otherwise identical polystyrene core, we decided to include a tri-β-Ala spacer between the aminomethyl Merrifield core and the BAL linker. However, after synthesis of the linear peptide sequence, the HR MAS NMR spectra again indicated that the *O*-allyl group could not be removed by treatment with the palladium catalyst and phenylsilane for 15 min. Therefore, we returned to the direct attachment of the BAL linker to aminomethylated Merrifield resin (Figure 1), and the reaction time for the deprotection was increased to 3 h. Surprisingly, in the HR MAS NMR spectrum all signals of the peptide had vanished and only the signals of the solid support remained (Figure 5). No cleaved peptide could be detected by HPLC-MS analysis of the filtrate of the deprotection reaction. When the reaction sequence was continued by Boc-deprotection, cyclisation and HF cleavage, as described before, the cyclic peptide could be isolated with a crude yield of 28–40% and HPLC purity above 95%. This indicates that despite the signals were not visible in the HR MAS NMR, the peptide was still grafted to the resin after the allyl deprotection step. The addition of $Pd(PPh_3)_4$ to the sample still containing the allyl ester did not influence the spectrum, excluding that a paramagnetic effect of Pd would be responsible for signal vanishing. Extensive line broadening and signal attenuation in HR MAS NMR spectra of resin-bound peptides have been reported before [6,13] and was shown to be linked to peptide aggregation, thereby limiting the rotational mobility of the peptide.

A series of experiments using aminomethylated Merrifield resin (loading 0.45 mmol g^{-1}) was carried out in order to explain and to confirm the above-assumed interpretation of HR MAS signal vanishing due to intra- or interchain aggregation. In order to obtain well isolated signals in the HR MAS NMR spectrum, the synthesis of the dipeptide Ile-β-Ala using the BAL strategy on aminomethyl Merrifield resin was chosen as a test (Scheme 2). Both Boc- and



Scheme 2. Synthesis of the dipeptide Ile-βAla to confirm the hypothesis that the disappearance of the HR MAS NMR signals is due to reduced mobility of the grafted dipeptide.

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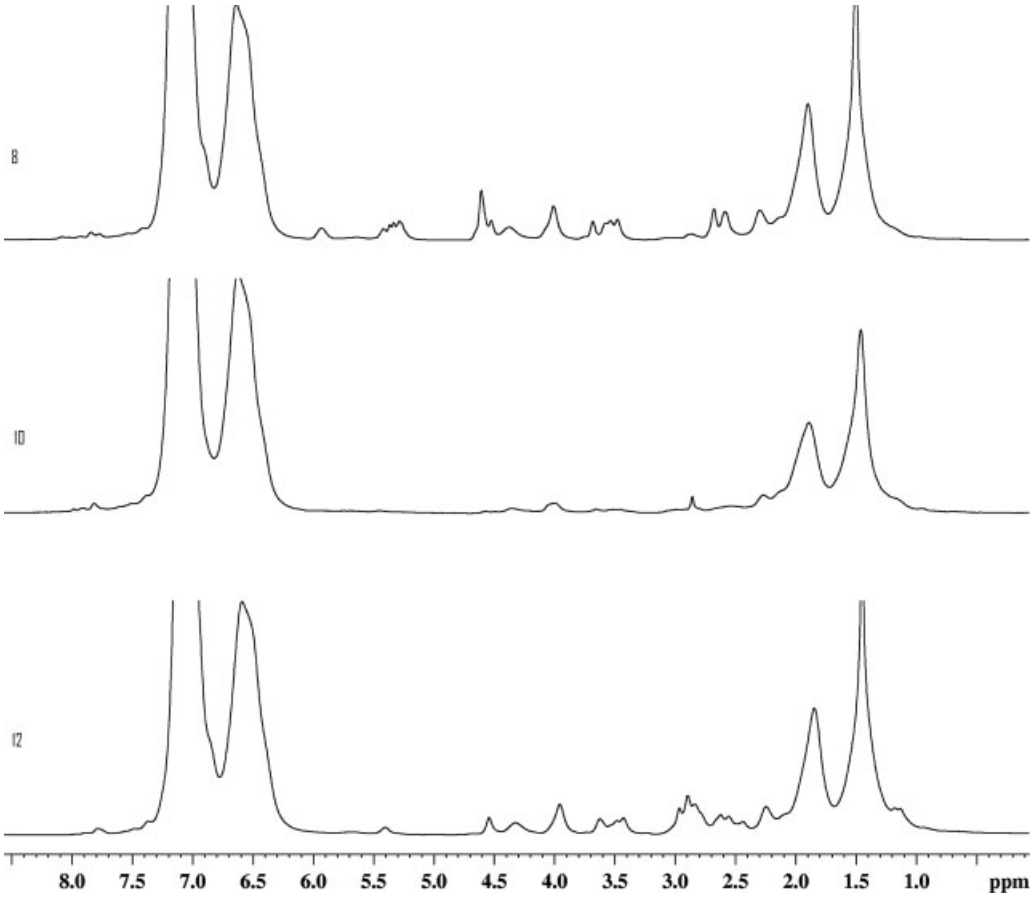

Figure 6. HR MAS spectra in $CDCl_3$ of the experiments to confirm the hypothesis of inter- and intrachain aggregations of the peptide chains: (8) coupling of Boc-Ile, (10) allyl deprotection (12) coupling of *N*,*N*-dimethylamine. The numbers correspond to the compound numbers in Scheme 2.

Fmoc protections were used. β-Ala-*O*-allyl ester was synthesised by the method of Nicolaou *et al*. [26] and attached to the solid support by reductive amination, followed by coupling of Ile as described above.

Again, all HR MAS NMR spectra showed the expected signals, until *O*-allyl removal caused the complete vanishing of virtually all peptide signals. In the case of the Fmoc-protected dipeptide, the loading of the resin could be determined by quantitative measurement of the dibenzofulvene-piperidine adduct during the Fmoc-deprotection step. This indicated that the peptide was still attached to the resin (0.41 mmol g^{-1} present starting from 0.45 mmol g^{-1}).

In order to find out whether the generated carboxylic acid is causing the loss of HR MAS NMR signal through its potential to be involved in hydrogen bonding via its OH group, *N*,*N*-dimethylamine was coupled to the free carboxylic acid in order to block any possibility to hydrogen bond interactions. As expected, this restored the usual HR MAS NMR spectrum of grafted peptide, its signals being visible again, (Figure 6) thus supporting the hypothesis that the vanishing was due to a reduced mobility. It is likely that strong hydrogen bonding involving somehow the carboxylic acid moiety is causing this. We tentatively propose that such a hydrogen bond could exist between the carboxylic proton and a carbonyl hydrogen bond acceptor of a neighbouring chain, resulting in loss of conformational mobility by cross-linking, but no evidence using any technique, in particular IR spectroscopy, could confirm this.

Conclusions

The main conclusion of this work is that HR MAS NMR is very useful for optimising reaction conditions during SPPS, even though loss of conformational mobility arising from any interchain aggregation involving peptide chains can lead to dramatic signal vanishing of HR MAS NMR resonances.

Acknowledgements

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