Single-bead structure elucidation. Requirements for analysis of combinatorial solid-phase libraries by Nanoprobe MAS-NMR spectroscopy

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A complete NMR structure analysis of an eight-residue peptide, covalently bound to a single-bead of a poly(ethylene glycol) based (POEPOP 1500) resin, is described. Well resolved 1D and 2D ^{1}H NMR spectra were obtained using magic angle spinning (MAS) Nanoprobe NMR spectroscopy at 500 MHz. The quantity of peptide on individual beads was determined after each NMR experiment by cleaving the peptide from the resin, and subsequent measurement of the fluorescence from the Abz-containing peptide in solution. It was found that about 1.6 nmol of peptide was the minimum amount needed to obtain a high resolution 1D ^{1}H NMR spectrum in less than 20 minutes. For a complete structure elucidation by 2D ^{1}H NMR a loading of about 6 nmol of peptide on a single-bead was required. It has been demonstrated that, with a sufficient loading on PEG-based resins, a complete structure elucidation of a resin bound octapeptide on a single bead can be achieved. This observation may be useful for screening and analysis of 'one-bead, one-compound' libraries.

Introduction

In the last few years, there has been a surge in interest in the study and development of solid-phase combinatorial organic synthesis.[1] This technique, by which large numbers of compounds are rapidly synthesised in concert with the emergence of high-throughput screening methods, allows the fast identification of new leads for targets in areas of drug development, material sciences and catalysts.[2] A limitation of solid-phase synthesis lies in the characterisation of products, or intermediates, while still covalently bound to the resin.[3] Currently, the products are cleaved from the resin, and subsequently analysed using conventional techniques. However, there is considerable interest in developing analytical techniques for characterising samples while they are still covalently attached to the resin. A non-destructive technique would prove particularly useful in the rapidly expanding field of 'one-bead, one-compound' library methods.[4] From a diverse library of possibly millions of individual beads, only one bead carrying the active compound may be isolated and the success of this approach depends upon determining the structure of the compound bound to a single bead.[5]

New mass spectrometry (MS) techniques have recently been developed which can analyse single-bead samples without the use of encoding ligands. However, all such methods are destructive.[6] Fourier transform infrared spectroscopy (FT-IR) has been used successfully in monitoring reactions, but is of limited utility for a full structure analysis since it only indicates the presence of distinct functional groups.[7]

The advances in pulse sequences and development of magic angle spinning (MAS) nano- or high resolution-NMR probes, have made possible studies of resin bound material by NMR.[8] Through the incorporation of ^{13}C-labelled building blocks near the reaction site, ^{13}C-NMR has proven useful for monitoring the reaction.[9] However, the use of specially labelled building blocks is of limited utility due to their restricted availability and high cost. Proton NMR has also been used to monitor reactions on solid phases in single-bead detection using a MAS-NMR probe.[10] Although, single-bead detection was achieved, the amount of material needed for a full structural characterisation was not determined, and the structural analysis of the compound was limited to confirmation of each synthetic step in a known sequence of reactions. The possibility of performing a structural analysis using MAS-NMR on solid-phase bound peptides and monitoring the addition of amino acids has been reported using a Wang resin and 10 mg of beads for the analysis.[11]

The requirements for a full structural analysis of a given compound depend on its size, chemical composition and the NMR properties of the polymer matrix used for the solid-phase synthesis. For peptides or peptoids it is crucial to get the sequential connectivity assignment from NOESY spectra, whereas with other types of compounds COSY and TOCSY provides most of the necessary information.

As part of a program to develop analytical methods for solid-phase organic combinatorial chemistry, the successful use of ^{1}H NMR Nanoprobe technology to obtain a full structural characterisation of a resin bound peptide on single beads is reported. The peptide is referred to as an eight-residue peptide, including six normal amino acids, a linker, and a 2-aminobenzamide (Abz) group attached to a single resin bead. The sequence and composition were unknown to the spectroscopist. The amount of peptide on the individual beads was determined by fluorescence spectroscopy, utilising the fluorescence properties of the N-terminal Abz group.

Results and discussion

The polymer matrix (POEPOP 1500) used in this study was selected due to its favourable spectroscopic properties,[12] as it produced well resolved NMR spectra, with narrow line widths, for both the organic moiety and the polymer matrix. Furthermore, it shows good swelling properties in several different solvents.[12] These properties are essential when performing

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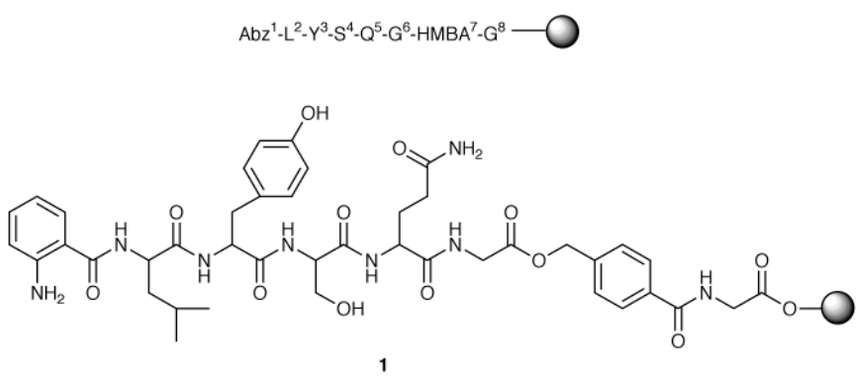

Fig. 1 The structure of the solid-phase bound peptide (**1**).

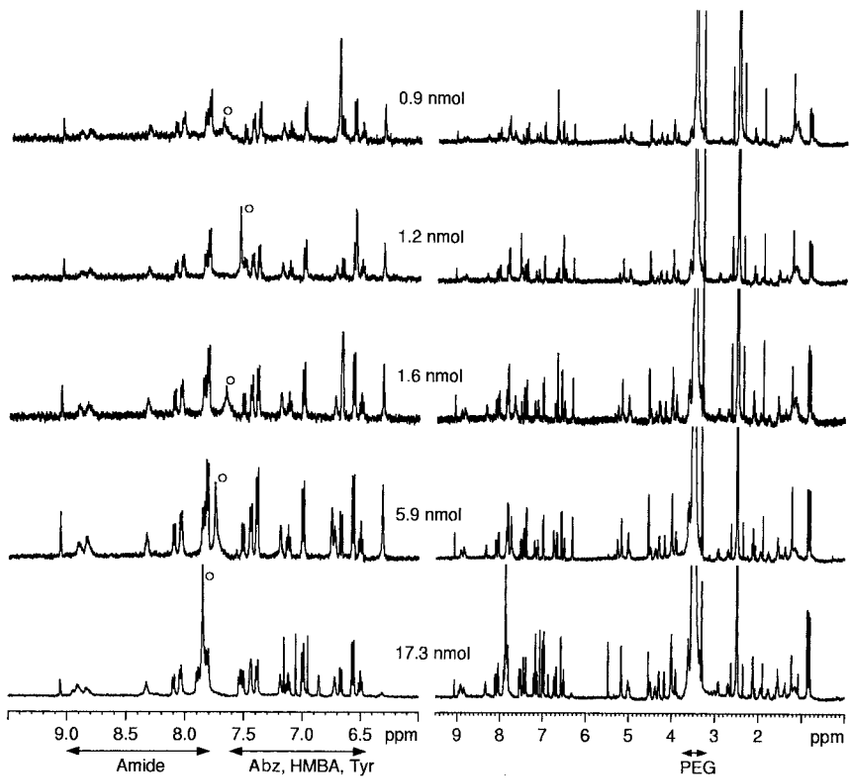

Fig. 2 1D ^{1}H NMR spectra of resin bound compound **1**. The amount of peptide present on the bead is shown on the spectra. All spectra were obtained with 256 scans except for the spectrum of 0.9 nmol, which is 512 scans. A total of 18 samples containing one bead were examined in order to see the variance in the data due to differences in bead size. The ○ marks peaks originating from spinning side bands.

both on-bead screening and analysis. Furthermore, the resonances originating from PEG did not interfere with the peptide assignments.

The solid support (POEPOP 1500) was synthesised by bulk polymerisation.[13] After polymerisation the material obtained was ground and sieved to particles of size 300–500 μm. These particles, referred to as beads, are of different sizes and shapes due to their preparation. The resin was functionalised with a 4-hydroxymethylbenzoic acid (HMBA) linked *via* a glycine residue, and used for standard solid-phase peptide synthesis (SPPS) of an eight-residue peptide **1** (Fig. 1). Upon completion the resin was treated with aqueous TFA to remove all the side chain protecting groups. A portion of the resin was treated with aqueous NaOH to cleave the peptide (peptide without the linker and a Gly) from the support. The purity (>93%) was determined on an analytical RP-HPLC column. Subsequent Edman degradation and MALDI-MS confirmed the expected amino acid sequence. The sequence was not revealed to the part of the group performing the NMR assignment.

Single-bead samples were prepared in a nanotube as described in the Experimental section. In order to investigate the amounts of sample required to obtain a 1D ^{1}H NMR spectra, several spectra were recorded on beads of varying size. Each of the 1D ^{1}H MAS-NMR spectra shown in Fig. 2 were acquired from single-bead samples. In most cases, good 1D ^{1}H NMR spectra could be acquired in about 17 minutes with 256 scans and a repetition time of 4 s. The overall spectral quality and the amount of peptide bound are shown in Fig. 2 and Table 1. The rather broad size distribution of the beads was reflected in the large variation in the amount of material present on each bead. The quantity of peptide on individual beads was determined after each NMR experiment by cleaving the peptide from the resin and subsequent measurement of the fluorescence from the Abz-containing peptide in solution, and by comparison to a standard curve as described in the Experimental section.

A sequential assignment through the peptide chain requires detection of the labile amide resonances. In the spectra acquired with less than 1.6 nmol some amide resonances disappeared, probably due to an increase in the exchange rate of the amide protons with residual water present in the DMSO-d_6 (Fig. 2). A bead containing 1.6 nmol of peptide gave a satisfac-

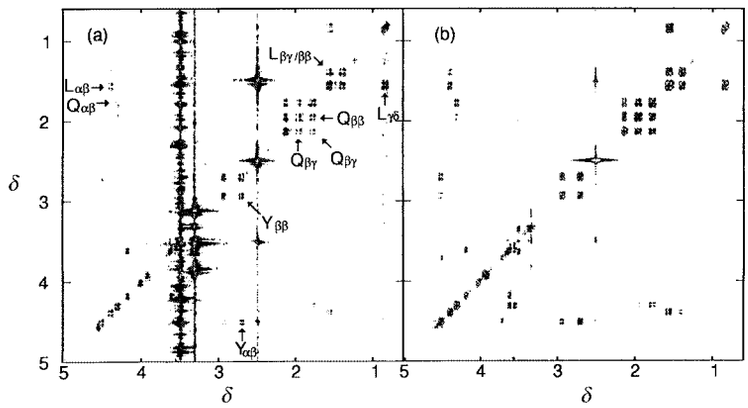

Fig. 3 DQF-COSY 2D spectra for compound **1** (a) 5.9 nmol sample, (b) 28.4 nmol sample. Indicated on (a) are the cross peak assignments of the Leu, Gln and Tyr side chains.

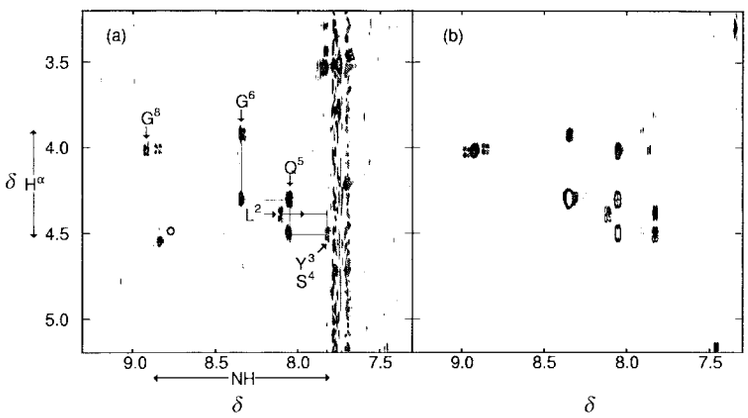

Fig. 4 NOESY-DQF COSY overlay of the amide-H^α area for (a) 5.9 nmol sample, (b) 28.4 nmol sample. On (a) the sequential assignment path is indicated. The ○ marks peaks originating from spinning side bands along the diagonal.

Table 1 Quantification by fluorescence ($\lambda_{em} = 420$ nm) of peptide amounts present on the single beads

Sample number	Fluorescence intensity	[Peptide]/μM	Amount of material/nmol
1	4.56	1.06	0.9
2	6.46	1.51	1.2
3	8.47	1.98	1.6
4	30.80	7.19	5.9
5	84.37	19.69	17.3

tory spectrum with all resonances present. The spectrum obtained on the 17.3 nmol sample only shows an improved signal to noise ratio when compared to the 1.6 nmol sample.

However, to obtain the sequential assignment a 2D spectrum was needed. To acquire a 2D data set in a reasonable time a higher loading than 1.6 nmol was required. A full assignment of the amino acids was obtained from 2D NOESY and DQF-COSY spectra [14] on a 5.9 nmol single-bead sample. The NOESY and DQF-COSY spectra used for the assignments were acquired using 48–64 scans for each increment. The NOESY spectrum of the 5.9 nmol sample was acquired in 17.4 hours and the DQF-COSY spectrum in 17.5 hours.

The individual amino acid resonances were assigned in the DQF-COSY spectrum of the 5.9 nmol sample by using their characteristic spin–spin coupling patterns. The resonances of the tyrosine, leucine and glutamine (Fig. 3a) as well as those of the HMBA (data not shown) and the Abz group (data not shown) could readily be identified. However, the serine resonances were more difficult to assign due to complete overlap between the Tyr H^α and the Ser H^α resonances as well as their respective amide (NH) protons (Fig. 4a). In addition, the serine H^β resonances were most likely close to the H^α resonance, which made the assignment difficult. To obtain the sequential order of the amino acids a NOESY spectrum of the 5.9 nmol sample was acquired. The identified $H^\alpha(n)$–NH(n) connectivities obtained from the DQF-COSY spectrum were linked using the $H^\alpha(n)$–NH(n + 1) connectivity visible in the NOESY spectrum. In Fig. 4a (5.9 nmol sample) the H^α–NH areas of the DQF-COSY and the NOESY spectra are overlaid and the sequential path can be followed, starting with the intraresidue leucine NH–H^α connectivity and continuing through five of the amino acids in the 5.9 nmol sample. There were no discontinuations in the connectivity pattern for the one-bead sample, but the presence of the sequential NOE between the Leu–Tyr and Tyr–Ser was hampered by the presence of PEG-derived spinning side-bands close to the amide resonances (Fig. 4a). Spectra obtained at different spinning speeds of the nanotube, leading to a shift of the spinning side bands, demonstrated that all connectivities were present. The chemical shift assignments are given in Table 2.

After completing the assignment of the peptide on the 5.9 nmol sample a set of DQF-COSY and NOESY spectra were acquired on a sample with a higher peptide concentration (28.4 nmol on 4 beads). These spectra were used for verifying the structural analysis and the assignment of the serine H^β resonances. Evaluation of the 28.4 nmol DQF-COSY spectrum showed that the H^β protons could be located either close to the H^α resonance, or close to the main PEG resonance. The acquired 28.4 nmol spectra also verified that all important resonances and NOE connectivities were present in the spectra of the sample containing only 5.9 nmol of the octapeptide.

Proton NMR solution spectra of a small amount of the cleaved peptide were subsequently acquired in the Nanoprobe. The spectra acquired in DMSO-d_6 indicated that the peptide

Table 2 Chemical shifts for compound **1** [a]

	δ				
Residue	NH	αH	βH	γH	δH
Gly (−1)	8.91	4.03	—	—	—
HMBA	H2,6 7.90/7.85/7.82 [b]	H3,5 7.54/7.44/7.38 [b]	—	—	—
Gly	8.34	3.91	—	—	—
Gln	8.04	4.29	1.76/1.95	2.13	—
Ser	7.81	4.49	4.60/3.70	—	—
Tyr	7.81	4.50	2.69/2.93	H2,6 7.00	H3,5 6.56
Leu	8.10	4.38	1.38/1.55	1.55	0.81/0.85
Abz	H2 7.52	H3 6.50	H4 7.13	H5 6.67	—

[a] Reference to DMSO at 25 °C, 2.49 ppm. [b] Several close-lying HMBA spin systems are seen in the aromatic region.

adopts two major conformations. This was based on two possible assignments of sequential paths through the peptide backbone, obtained from 2D spectra recorded at both 25 and 40 °C (data not shown). The solution results are in contrast to those obtained from on-bead analysis, where only one sequential pathway could be found. The presence of two sequential pathways in solution was subsequently confirmed by an additional independent peptide synthesis, where different coupling conditions, linker and cleavage methods were used, to rule out the possibility of racemisation. Furthermore, an EI-MS spectrum showed only one peak at 686.5, pointing towards the presence of only one product. Finally, it was shown that upon a change in solvent H_2O–D_2O (9 : 1 v/v) only one major set of resonances was observed, indicating that the formation of multiple conformations in DMSO may be due to hydrogen bonding which is favoured in DMSO.

The peptide cleaved from the resin gave rise to additional resonances in both the DMSO and H_2O–D_2O (9 : 1 v/v) spectra, indicating two conformations. Addition of CF_3COOD to the latter solution sharpened the peaks indicating that the difference between the two solution peptide samples is most likely due to differences in salt concentrations and pH. Only minor differences were observed when comparing the chemical shifts of the peptide in solution with those attached to a bead.

In conclusion, it has been demonstrated that sufficient sensitivity is available to obtain a complete *de novo* structure analysis of an eight-residue peptide attached to a single bead at 500 MHz when an appropriate resin is used. With a loading of 0.45 mmol g^{-1} of the POEPOP 1500 resin and with a bead size of 300–500 μm it was found that well resolved 1D ^{1}H NMR spectra could be obtained on a bead with a loading of about 1.6 nmol for an eight-residue peptide. To obtain good quality 2D spectra, required for structure determination in a reasonable time, a higher loading of ~6 nmol was sufficient. This amount will eventually decrease with higher field NMR spectrometers, the use of gradients and increased probe sensitivity.

In addition, it has been demonstrated that using the fluorescent Abz-group as a sensitive marker is an easy way to quantify the amount of peptide present on a single bead.

Experimental

Resin synthesis

POEPOP 1500 resin was prepared as previously described [13] by anionic polymerisation of epoxy methyl-PEG 1500 ether (0.7 equiv. epoxide, 25 g) and *t*-BuOK (0.2 g, 1.64 mmol) without any solvent at 130 °C. The sticky point was reached after 20 min. The polymerisation was allowed to continue for a period of 12 h at 130 °C and then cooled to room temperature. The reaction was quenched with methanol (5 cm^3), swollen in DCM (175 cm^3) and granulated through a 1 mm sieve. Subsequently the resin was washed with methanol (4 × 100 cm^3), filtered and stirred in 4 M aqueous HCl over night. The acid was removed by filtration and the resin washed with water (6 × 100 cm^3), methanol (6 × 100 cm^3), DMF (6 × 100 cm^3) and DCM (6 × 100 cm^3) before it was dried by co-evaporation with dry acetonitrile (2 × 100 cm^3) and left *in vacuo* overnight. The dried resin (21.9 g, 88%) was granulated through a 500 μm sieve and small particles were removed with a 300 μm sieve. The hydroxy group capacity of the resin was 0.45 mmol g^{-1} as determined by esterification with Fmoc-Gly (3 equiv.), activated by 1-(mesityl-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT) (3 equiv.) in the presence of *N*-methylimidazole (2.5 equiv.) in DCM. Subsequently the UV absorbance of the adduct between dibenzofulvene and piperidine formed by treatment of a weighed polymer sample with 20% piperidine in DMF, was measured.

Peptide synthesis

POEPOP 1500 resin (200 mg, 0.45 mmol g^{-1}) was swollen in DCM and washed with DCM (3 × 2 cm^3). Fmoc-Gly-OH (120 mg, 0.4 mmol) was dissolved in dry DCM in the presence of *N*-methylimidazole (33 mm^3, 0.4 mmol) and activated with MSNT (120 mg, 0.4 mmol) before the solution was added to the resin. The reaction was left for 45 min at room temperature and the coupling was repeated. The resin was treated with 20% piperidine in DMF (4 cm^3) for 20 min and washed with DMF (6 × 2 cm^3) and DCM (6 × 2 cm^3). 4-*tert*-Butyldimethylsilyloxymethylbenzoic acid (120 mg, 0.45 mmol) was then attached to the support in the presence of 4-ethylmorpholine (NEM) (60 mm^3, 0.47 mmol) by *in situ* activation with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) (140 mg, 0.44 mmol).[15] The reaction was left for 2 h at room temperature. A negative Kaiser test showed complete reaction. The silyl protecting group was cleaved from the resin by treatment for 2 h with 1 M TBAF in THF–95% aqueous TFA (3 cm^3, 1 : 1 v/v). The resin was filtered, washed with DMF (6 × 2 cm^3) and DCM (6 × 2 cm^3), neutralised with 2% DBU in DMF (3 × 2 cm^3), washed with DMF (6 × 2 cm^3) and DCM (6 × 2 cm^3), and dried. Fluoren-9-ylmethoxycarbonylglycine (Fmoc-Gly) (120 mg, 0.4 mmol) was coupled to the resin by the MSNT esterification method as described above. The Fmoc group was cleaved and peptide synthesis was continued by using standard Fmoc-AA-OPfp (2.5 equiv.) ester couplings in the presence of 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (Dhbt-OH) catalyst (0.05 equiv.) and 20% piperidine in DMF for N^{α}-deprotections. Upon completion of the synthesis the protecting groups of the peptide were cleaved off while the peptide was still attached to the resin by treatment for 2 h with 95% aqueous TFA. The resin was filtered, washed with DMF (6 × 2 cm^3) and DCM (6 × 2 cm^3), neutralised with 2% DBU in DMF (3 × 2 cm^3), washed with DMF (6 × 2 cm^3) and DCM (6 × 2 cm^3), and dried.

Resin (5 mg) was dried under high vacuum and treated with 1.0 M aqueous NaOH (2 cm^3) for 2 h. The released peptide was filtered off and the resin washed with 1.0 M aqueous NaOH (2 cm^3) and water (2 cm^3). The combined washings and supernatant were neutralised with 1.0 M aqueous acetic acid and the solution was concentrated *in vacuo*. The crude product was

analysed by HPLC on a Delta Pak C18 (15 μ, 300 Å, 0.8 × 20 mm) with a gradient of MeCN in 0.1% aqueous TFA as eluent and a flow of 1 cm^3 min^{-1}. The crude compound eluted as a single major peak and was purified to homogeneity by HPLC chromatography affording 1.4 mg of pure peptide (93% yield based on initial loading by Fmoc monitoring). The pure peptide was characterised by MS; *m/z* 687.12 ($C_{32}H_{43}N_7O_{10}$ requires M + H = 685.82).

Resin (2 × 1 mg) was analysed by Edman degradation; Gly, 1.94 (2); Gln, 0.98 (1); Ser, 1.04 (1); Tyr, 0.99 (1); Leu, 1.03 (1).

A second peptide with identical sequence was synthesised on a 0.01 mmol scale using an ABI 432 Synergy Persional Peptide Synthesiser using standard Synergy amino acid columns and a Synergy pre-packed resin column in combination with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation.[16] Cleavage from the solid support and deprotection of the side chain protecting groups were carried out in TFA–ethane-1,2-dithiol–water (90 : 5 : 5 by volume, 5 cm^3) for 2 h at room temp. The filtrate was concentrated *in vacuo*. The crude product was purified by HPLC on a Delta Pak C18 (15 μ, 300 Å, 25 × 200 mm) with a gradient of acetonitrile in 0.1% aqueous TFA as eluent and a flow of 10 cm^3 min^{-1}. The crude compound was eluted as a single major peak and was purified to homogeneity by HPLC chromatography and afforded (14.6 mg, 88%) of pure peptide. The pure peptide was characterised by MS; *m/z* 686.5 ($C_{32}H_{43}N_6O_{10}$ requires M + H = 685.82).

NMR sample preparation

The beads were swollen in MeOH in order to separate them into single beads. The beads were subsequently dried overnight *in vacuo* in the nanotubes and suspended in 40 mm^3 of DMSO-d_6. In sample preparation the nanotubes were not totally filled and a small air bubble was always present when the tube was closed.

NMR experiments

All spectra were recorded on a Varian Unity Inova 500 MHz spectrometer equipped with a 4 mm ^{1}H-observe Nano NMR-probe. All spectra were measured at 25 °C with a spin rate of approximately 2 kHz. The 1D spectra were acquired as one-pulse experiments with presaturation of the remaining HDO resonance in DMSO. Acquisition data for the 1D spectra were as follows unless otherwise noted: 2.0 s acquisition time, 2.0 s presaturation delay, sweep width of 8000 Hz and sampled in 32000 points per 256 scans. All 1D spectra were processed using a 0.5 Hz line broadening and zero-filled to 64 K. NOESY spectra were acquired with mixing times of 250–300 ms, presaturation delay 1.4–1.5 s, 48–64 scans and 512 increments. The data matrix of 512 × 4096 was zero-filled to 1024 × 4096 prior to Fourier transformation by applying a shifted sine window function. DQF-COSY spectra were acquired with a presaturation delay between 1.3–1.5 s, 32–48 scans and 858–1024 increments. The data matrix of 858 × 4096 was zero-filled to 1024 × 4096 prior to Fourier transformation by applying a shifted sine window function. All spectra are processed in XWINNMR Bruker version 2.1. The presaturation period for the 5.9 nmol sample was divided between HDO and PEG.

Quantification

After evaporation of DMSO, 30 mm^3 of 1 M aqueous NaOH were added to each nanotube. After 2 h at room temperature the solution was transferred to an Eppendorf tube and the bead was washed with 1 M aqueous NaOH (2 × 30 mm^3). The combined washings and supernatant were neutralised with 1 M aqueous acetic acid (90 mm^3) and each sample were diluted with phosphate buffer (820 mm^3, 50 mM NaH_2PO_4–Na_2HPO_4, 2 mM cysteine, 1 mM EDTA, pH 6.5). The amount of peptide in each sample was determined by measuring the fluorescence from the Abz group at λ_{ex} 320 and λ_{em} 420 nm in a Perkin-Elmer LS50 spectrofluorometer. The peptide concentration was calculated using a standard curve (at λ_{em} 420 nm, intensity of 4.29 = 1 μM, R^2 = 0.997) produced from Abz-Gly-Ala-Gly-Ala-Phe-OH for the concentration range 1–100 μM. The fluorescence measurements were performed in a 0.1 cm^3 quartz microcuvette with 2.5 mm slit width for both excitation and emission. Repetition of the cleavage procedure on the same bead yielded no additional fluorescence.

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