Supplementary Material: Atomic resolution structure of a disease-relevant Aβ(1–42) amyloid fibril

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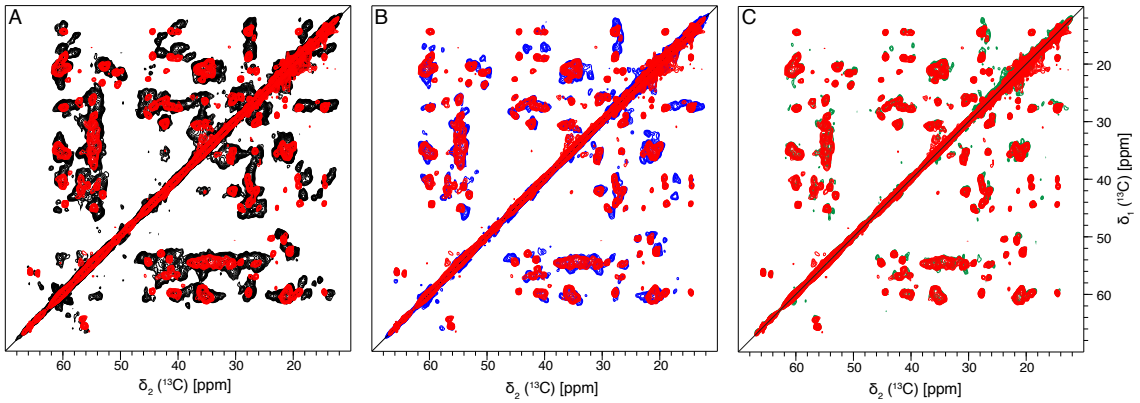

Fig. S1. Screening of conditions towards a sample with a single polymorph. Superposition of several 2D [^{13}C,^{13}C] DARR spectra (20 ms mixing time, 13 kHz MAS, 14 T B0) of Aβ(1-42) fibrils grown at different conditions. The condition 2 (red) is compared to condition 0 (black) (A), to condition 3 (blue) (B) and to condition 4 (green) (C). Condition 0 shows very broad lines and no clearly defined peaks. This condition shows at least 3 polymorphs.

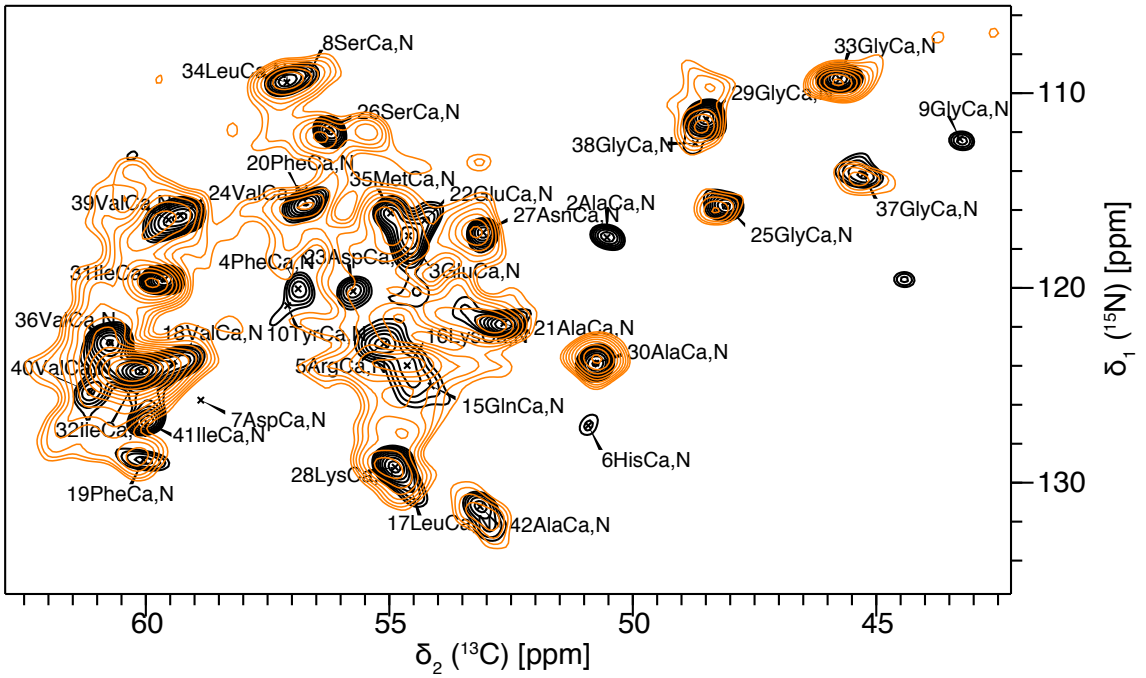

Fig. S2. Superposition of a NCA (plotted in black) and TEDOR (plotted in orange) spectra measured on uniformly labeled ^{13}C,^{15}N-labeled fibrils and 50% mixed labeled ^{13}C,^{15}N-labeled fibrils, respectively. Peaks in the NCA spectrum are labeled according to the assignment. The presence of superimposing cross-peaks between two spectra indicates an in-register parallel β-sheet structure.

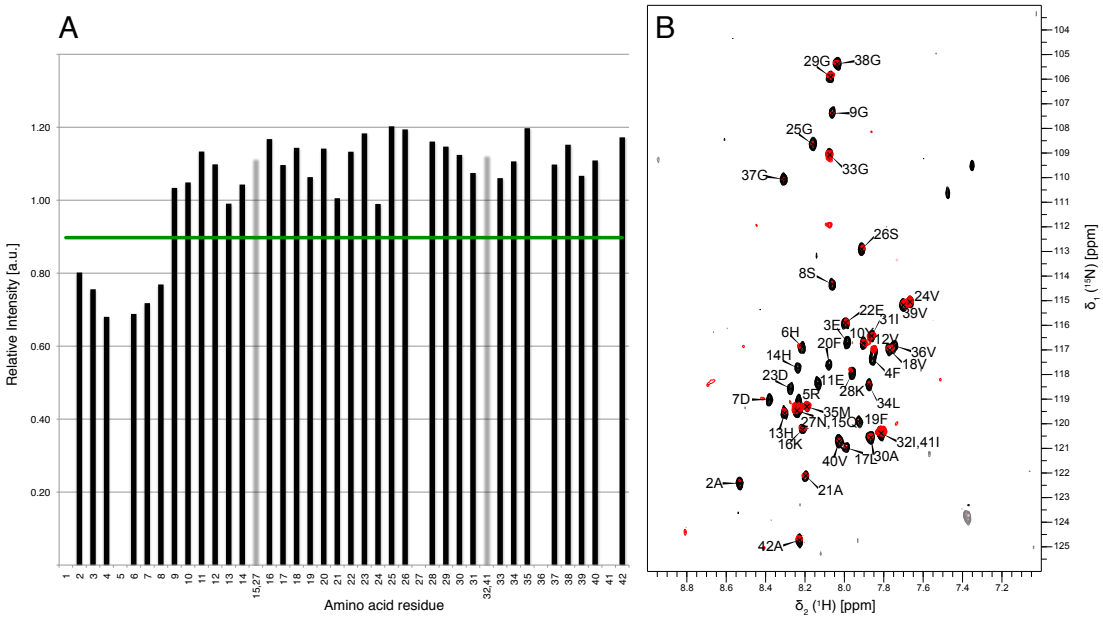

Fig. S3. Limited proteolysis by proteinase K of Aβ(1-42) fibrils grown under condition 2 indicating the heterogenous structural nature of its N-terminal segment. (A) The relative intensities of the ^{15}N-^{1}H moieties measured in a [^{15}N,^{1}H]-TROSY spectra of the digested fibrils are represented as bars in relation to the control sample (without digestion). In grey are data shown from overlapping cross-peaks. The green lines represent the median of the intensity ratios plus (upper) and minus (lower) the standard deviation of the measurements. (B) In black is shown the [^{15}N,^{1}H]-TROSY reference spectrum of Aβ(1-42) in 85% DMSO, 0.1% TFA and 10% H_2O recorded at 298 K on a Bruker 700 MHz spectrometer. The individual cross-peaks are assigned with the one letter amino acid code. In red, the corresponding [^{15}N,^{1}H]-TROSY spectrum is shown after overnight proteinase K digestion of the fibrils in buffer condition 2 at 37 °C in a ratio of 1:10 (w/w) proteinase K: Aβ(1-42).

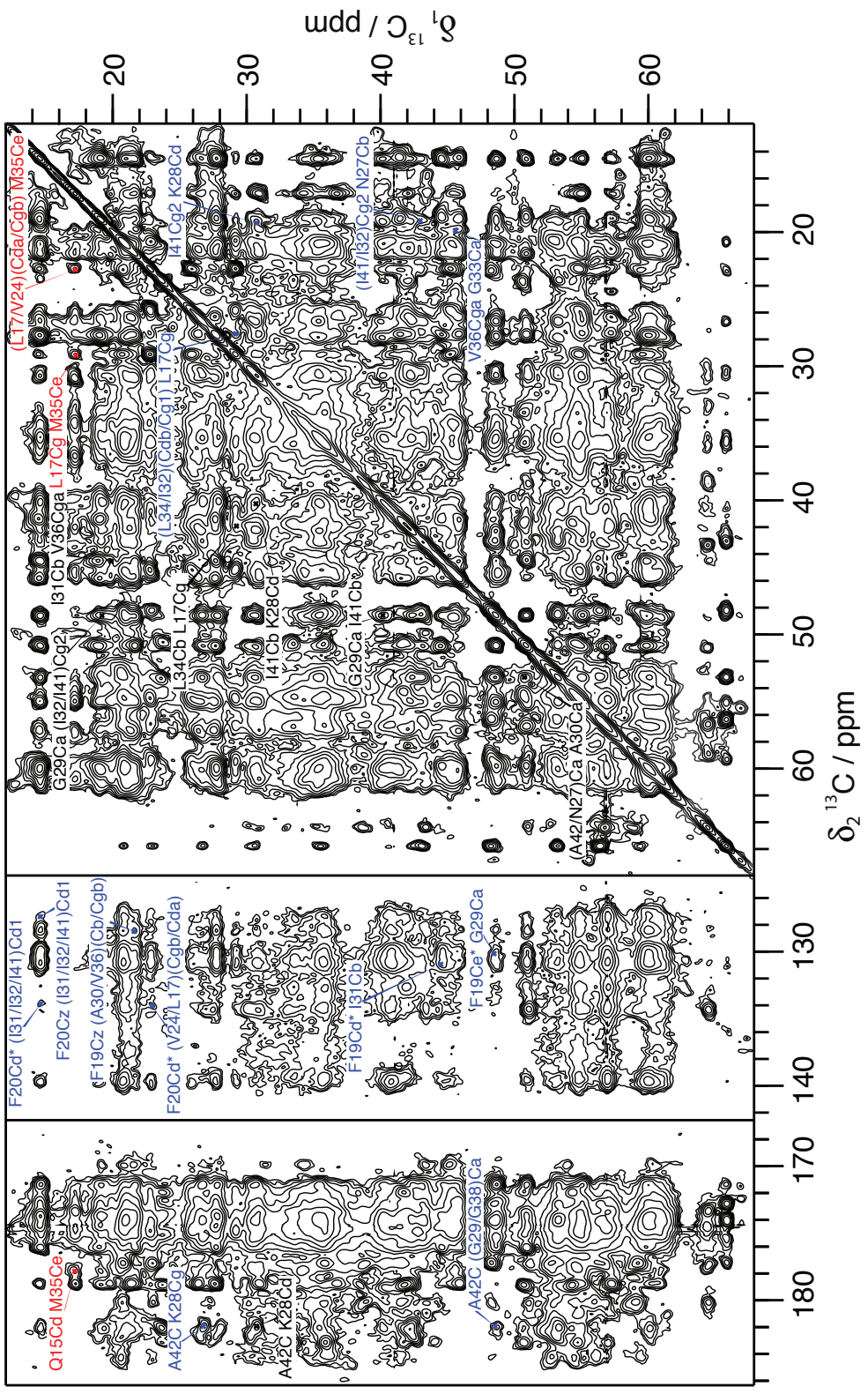

Fig. S4. 400 ms DARR spectrum as described in the main text. Cross-peaks listed in Table S2 and used in the calculation are marked by crosses and labeled in red (intermolecular restraints), black (intramolecular) or blue (ambiguous with respect to intra-/intermolecular).

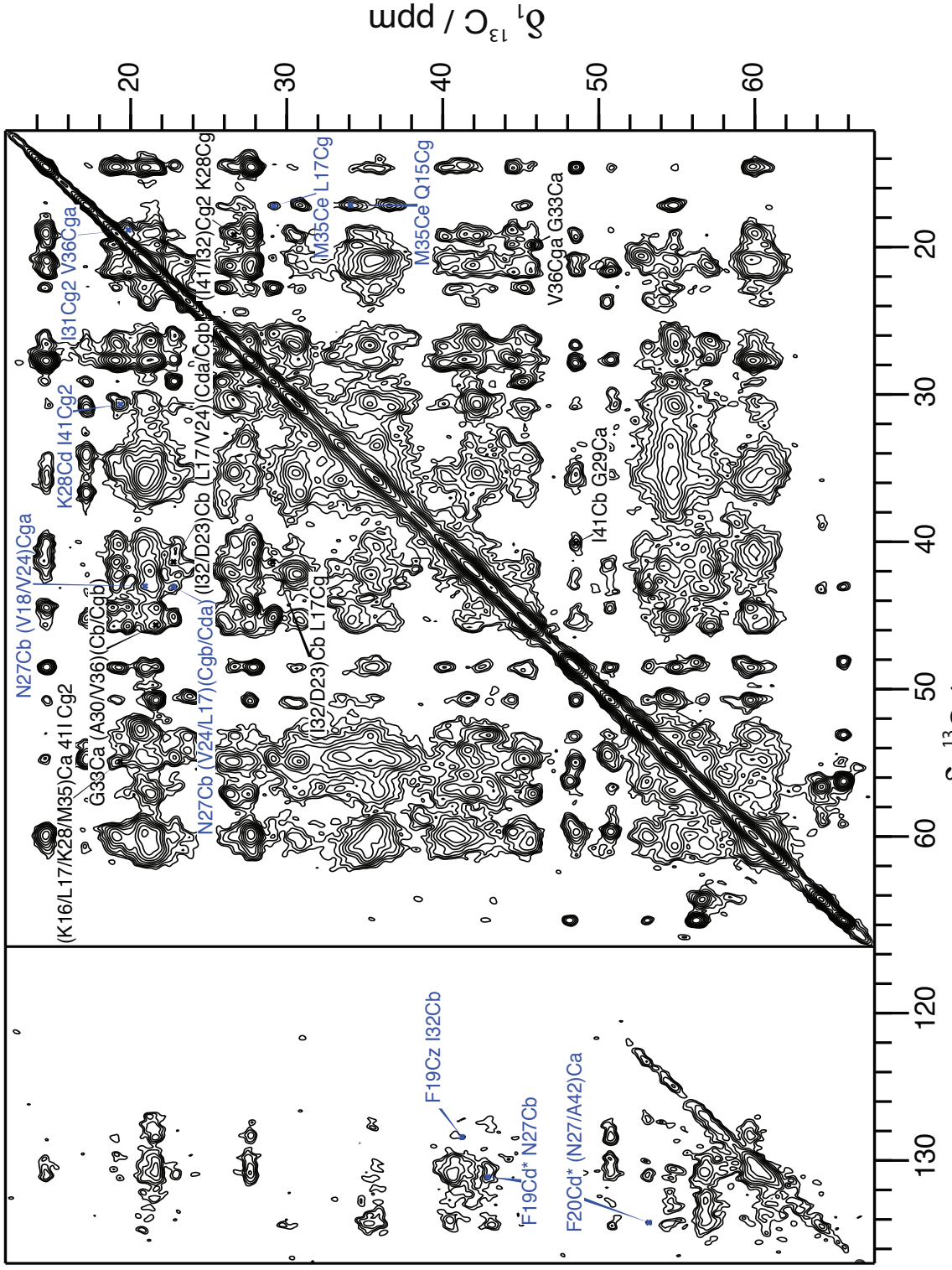

Fig. S5. 400 µs CHHC spectrum as described in the main text. Cross-peaks listed in Table S2 and used in the calculation are marked by crosses and labeled in red (intermolecular restraints), black (intramolecular) or blue (ambiguous with respect to intra-/intermolecular).

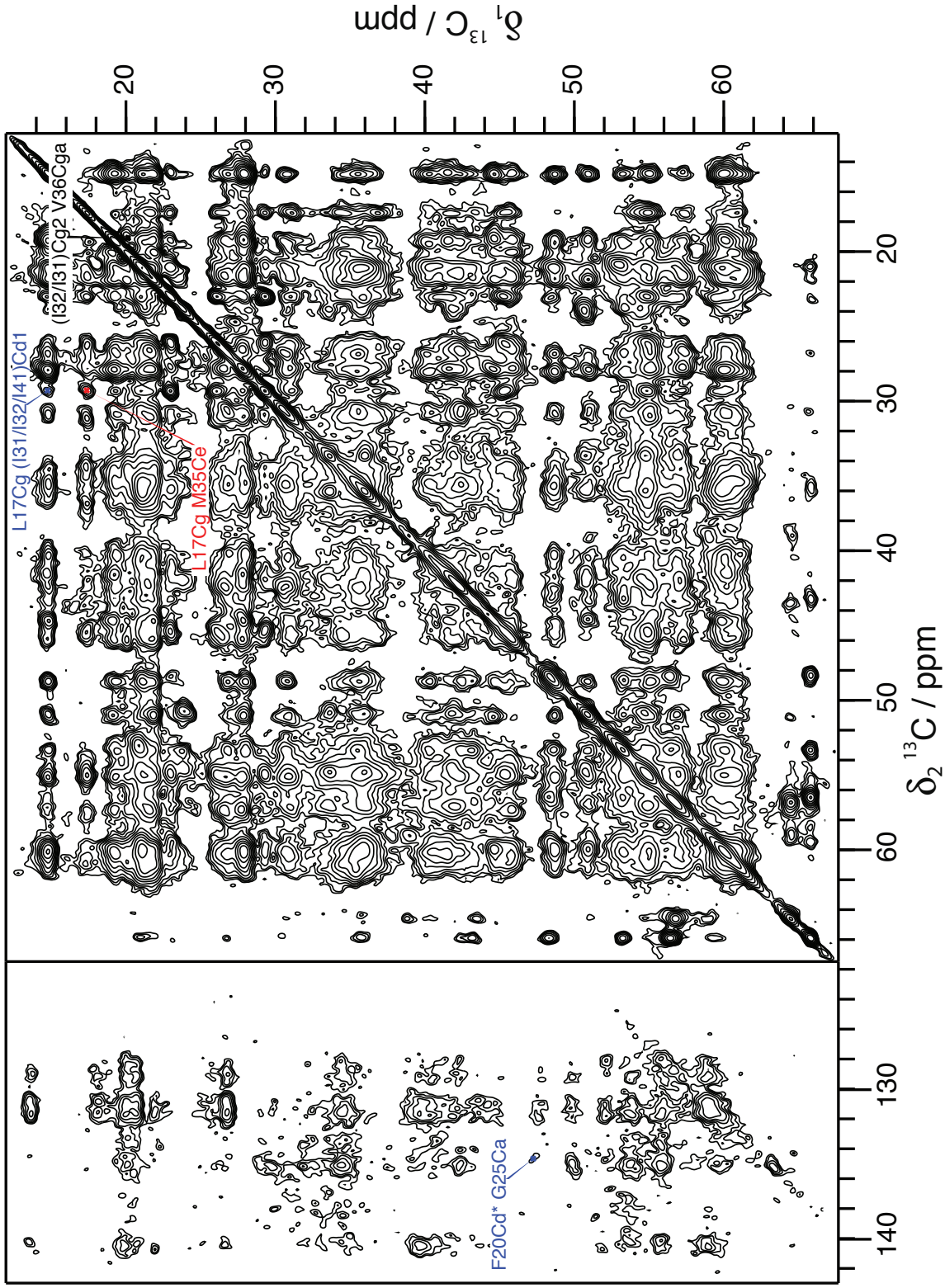

Fig. S6. 8 ms PAR spectrum as described in the main text. Cross-peaks listed in Table S2 and used in the calculation are marked by crosses and labeled in red (intermolecular restraints), black (intramolecular) or blue (ambiguous with respect to intra-/intermolecular).

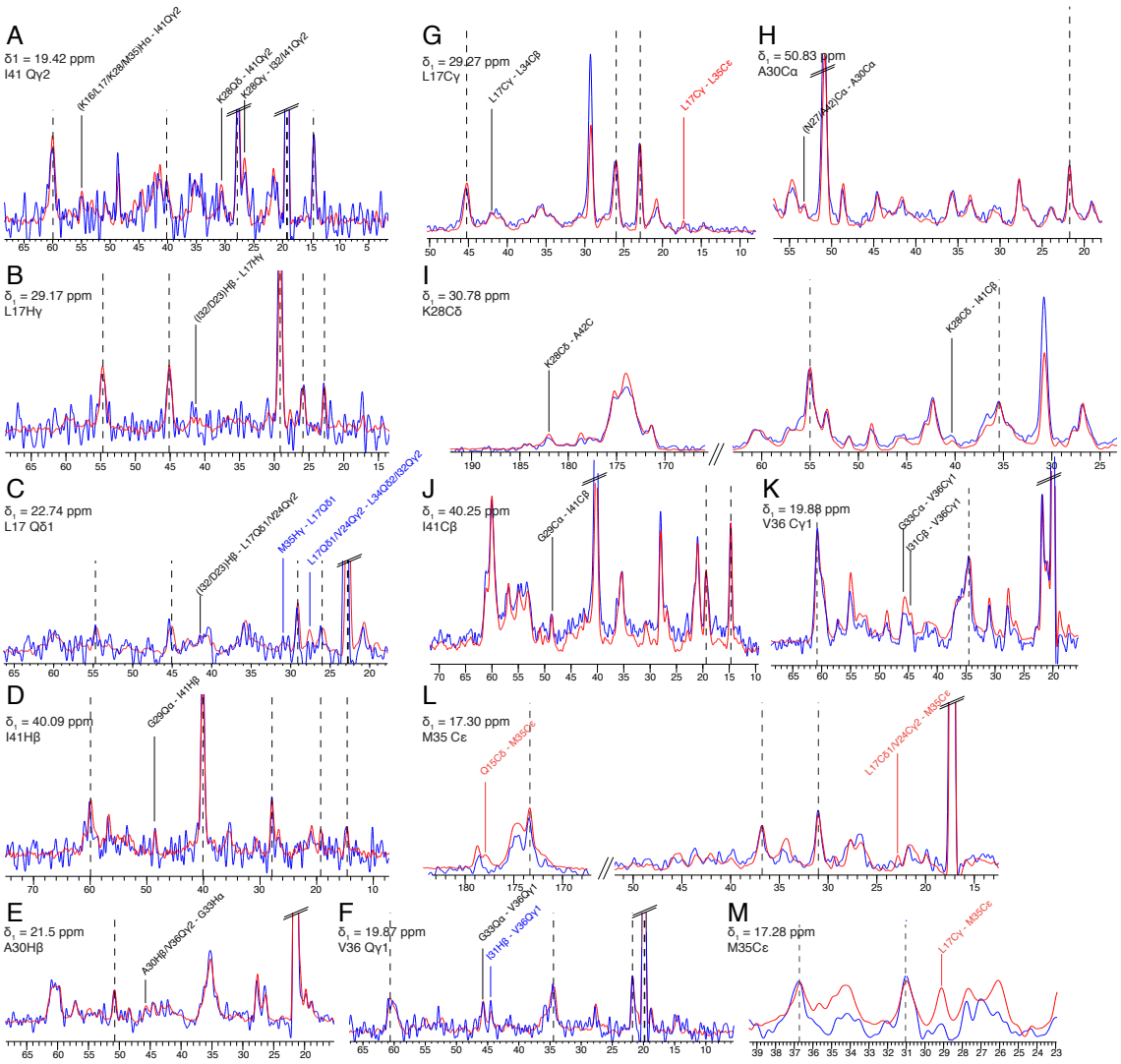

Fig. S7. Selected traces from the 400 μs CHHC (A-F), 400 ms DARR (G-L) and 8 ms PAR (M) spectrum of Aβ(1-42) fibrils. In red are the traces from the uniformly ^{13}C,^{15}N-labelled sample, while in blue are the corresponding traces from the diluted sample. The frequency of the trace together with the corresponding assignments are listed on the top left of each subfigure. Cross-peaks of interest are labeled black if they are considered to be intra-molecular, red inter-molecular, and blue if they are spectrally unambiguous intra- or intermolecular. The calibration of the diluted spectra was performed using the intra-residual peaks (black dashed line).

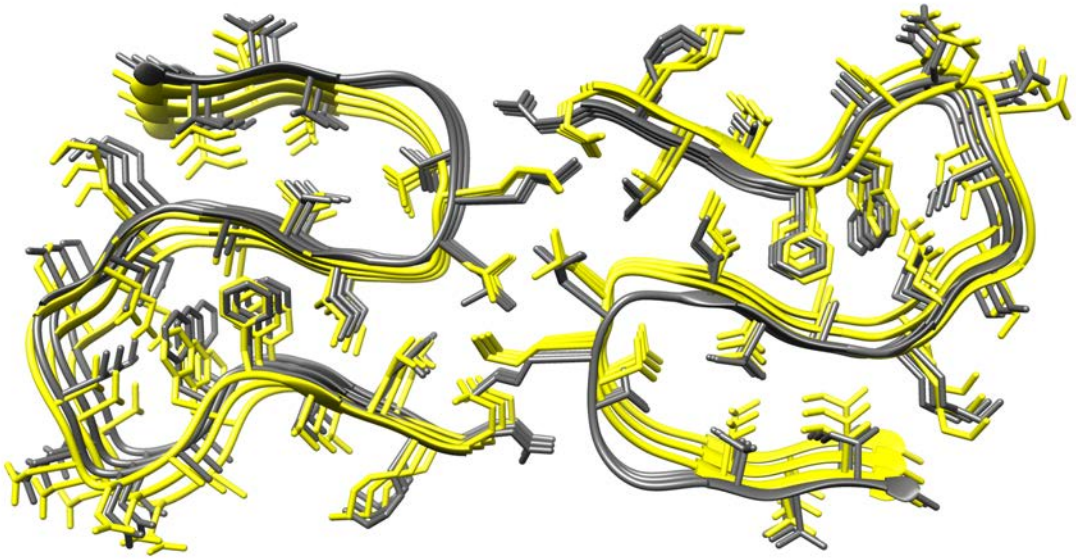

Fig. S8. Superposition of the 3D structures of the manual calculation and the automatic one. In yellow are 3 layers of the Aβ(1-42) fibrils using only manually assigned peaks, whereas in grey, automatically picked peaks are included to the structure calculation. The two structures superimpose well with an RMSD of 0.98 Å.

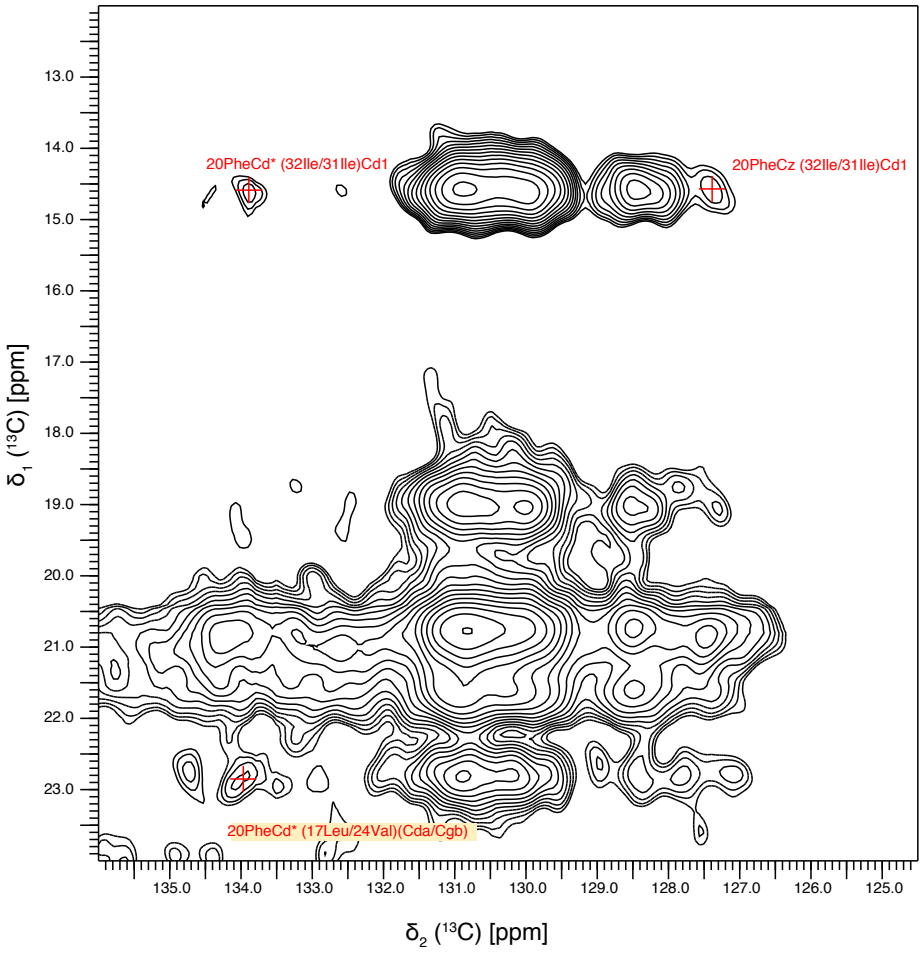

Fig. S9. Extract of the 400 ms DARR spectrum showing additional contacts demonstrating that F20 is oriented inside the hydrophobic core.

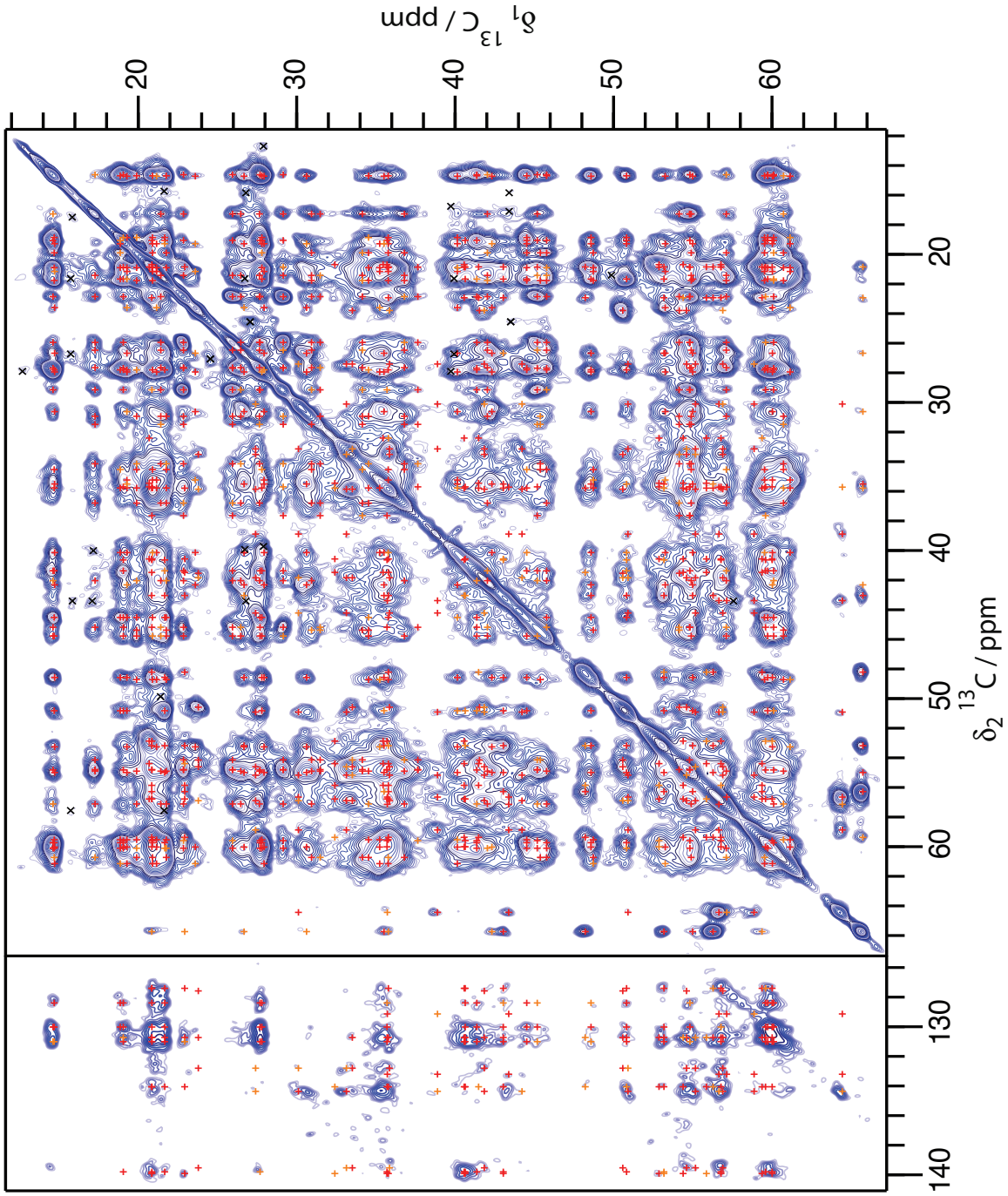

Fig. S10. Cross peak back-calculation from the determined 3D structure onto the PAR spectrum. Blue contours: aliphatic and aromatic regions of a PAR spectrum with 8 ms mixing time recorded on uniformly ^{15}N,^{13}C-labeled fibrils at 19 kHz MAS at a static magnetic field of 20.0 T (850 MHz proton frequency). Contacts between ^{13}C nuclei up to 7 Å were calculated from the structure of Fig. 4 and back-predicted onto the spectrum. Red crosses mark the expected peak positions. Contacts between 7 and 8 Å are marked in yellow. Black crosses mark remainders of a second polymorph. The back-calculation matches the experimental spectrum.

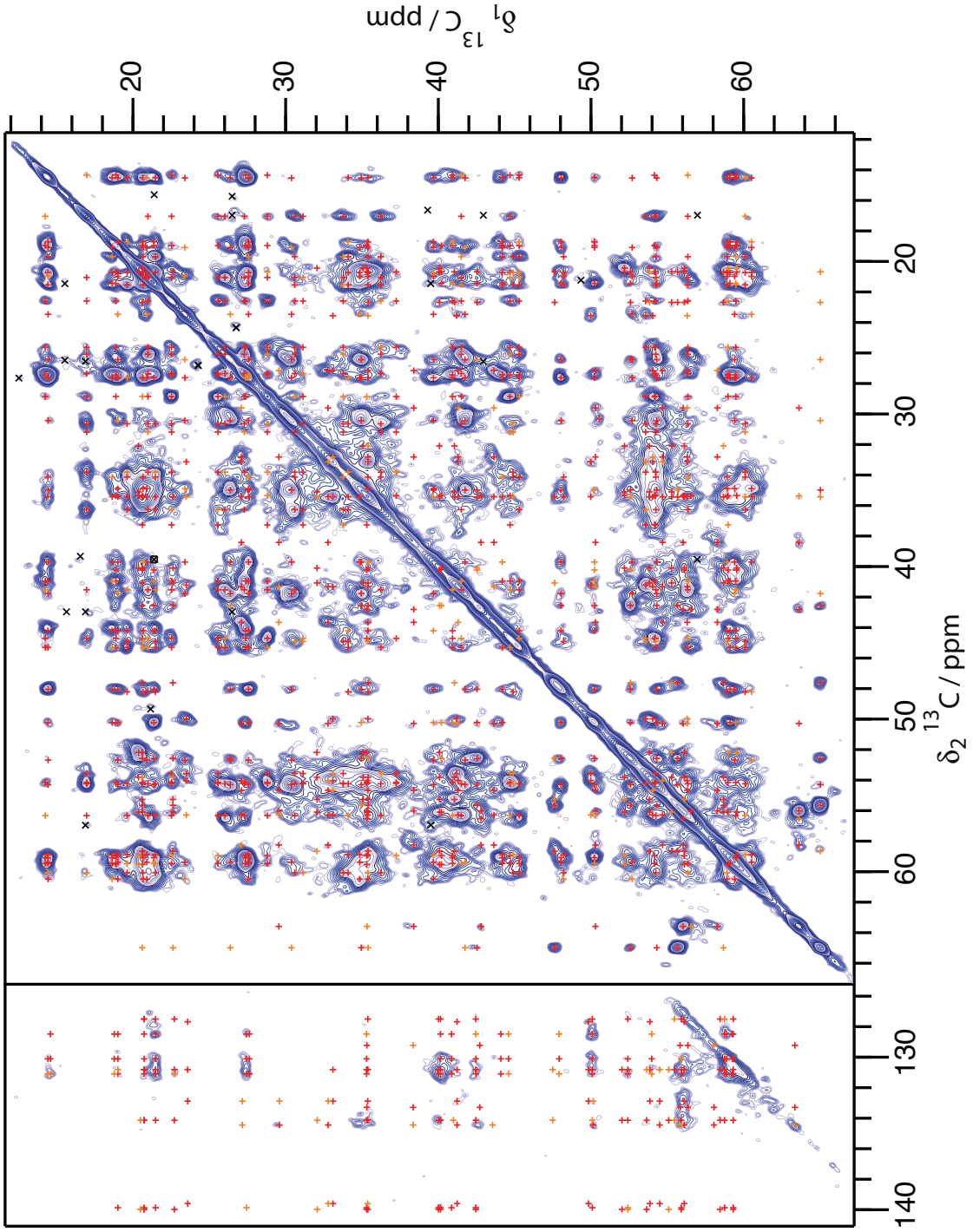

Fig. S11. Cross peak back-calculation from the determined 3D structure onto the CHHC spectrum. Blue contours: aliphatic region of a CHHC spectrum with 400 µs mixing time recorded on uniformly ^{15}N,^{13}C-labeled fibrils at 19 kHz MAS at a static magnetic field of 20.0 T (850 MHz proton frequency). Contacts between ^{13}C nuclei up to 5.5 Å were calculated from the structure of Fig. 4 and back-predicted onto the spectrum. Red crosses mark the expected peak positions. Contacts between 5.5 and 6.5 Å are marked in yellow. Black crosses mark remainders of a second polymorph. The back-calculation matches the experimental spectrum.

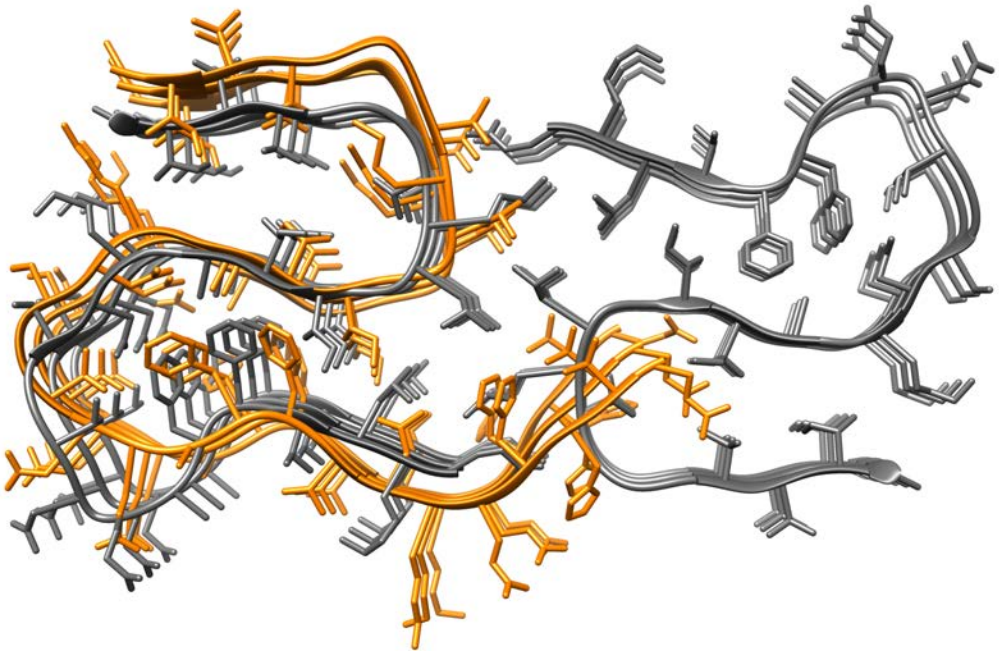

Fig. S12. Comparison of the 3D structures of the Aβ(1-42) fibrils from this study (grey) to a previously published structure (orange) (2mxu.pdb). The two structures do not superimpose well having an RMSD of 4.1 Å. Clear differences are found in the number of molecules per layer and in the side chain packing within the region of amino acid residues 34-36 for example.

Table S1: Condition screening of Aβ(1–42) fibrils including sample preparations of others.

Nr.	Seeding	A β species	A β con-centration [μ M]	Shaking	Temperature	buffer	Additives/Salt	Polymorphisms
0	No	recombinant A β (1-42)	150	gently	37 °C	100 mM phosphate pH 7.4	no	many
1	Yes	recombinant A β (1-42)	30	350 rpm	37 °C	100 mM phosphate pH 7.4	100 mM NaCl, 30 μ M heparin (~5kDa)	-
2	Yes	recombinant A β (1-42)	30	350 rpm	37 °C	100 mM phosphate pH 7.4	100 mM NaCl, 100 μ M ZnCl	1
3	Yes	recombinant	30	350 rpm	37 °C	100 mM phosphate pH 7.4	100 mM NaCl	~2
4	Yes	recombinant A β (1-42)	100	350 rpm	37 °C	100 mM phosphate pH 7.4	100 mM NaCl, 100 μ M ZnCl	1
Ishii and coworkers [27]	Yes	synthetic A β (1-42)	50	slow	room temperature	10 mM phosphate pH 7.4	no	1
Griffin and coworkers [54]	Yes	recombinant A β (M0,1-42)	10-50	n.d.	room temperature	20 mM sodium phosphate pH 8	no	1

Table S2. Manually assigned restraints for the structure calculation. In grey are the intra-molecular restraints, in pink the inter-molecular ones, and no color shows that the restraint cannot be classified as inter- or intra-molecular (and is used as ambiguous with respect to inter- or intramolecular in the calculations). Spectrally unambiguous restraints have a single entry, and spectrally ambiguous restraints have several entries reflecting the different assignment possibilities. One or two arrows in front of the entry indicate that the intramolecular or intermolecular assignment has been fulfilled in the structure calculation, respectively. The restraints have been collected from the PAR, the CHHC or the DARR spectra as indicated. Q indicates pseudoatoms that represent a group of degenerate hydrogen atoms. UPL indicates the upper distance bound used in Å, and d indicates the average distance in the final structure bundle.

PAR	ω1		ω2		UPL	d
➤	I31	CG2	V36	CG1	7.0	6.72
	I32	CG2				
➤➤	L17	CG	M35	CE	7.0	5.37
	L17	CG	I31	CD1		
➤			I32	CD1	7.0	4.47
			I41	CD1		

CHHC	ω1		ω2		UPL	d
	K16	HA	I41	QG2		
	L17	HA				
➤	K28	HA			5.5	3.19
	M35	HA				
	L17	HG	D23	HB		
➤			I32	HB	5.5	5.37
	L17	HG	I32	QD1		
➤			I32	QD1	5.5	2.77
			I41	QD1		
	L17	QD1	I31	QD1		
➤			I32	QD1	5.5	3.52
			I41	QD1		
	V24	QG2	I31	QD1		
			I32	QD1		
			I41	QD1		
	L17	QD1	D23	QB		
➤			I32	HB		
➤	V24	QG2	D23	QB	5.5	5.07
			I32	HB		
➤	N27	QB	A30	QB	5.5	2.36
			G9	QA		
	K28	QG	I32	QG2		
➤			I41	QG2	5.5	2.48
➤	29	QA	I41	HB	5.5	2.71
➤	G29	QA	I41	QG2	5.5	2.01
	A30	HB	G33	QA		
➤	V36	QG2			5.5	2.22
➤	G33	QA	V36	QG1	5.5	4.21
➤➤	Q15	QG	M35	QE	5.5	2.96
	L17	QD1	N27	QB		
➤	V24	QG1			5.5	4.91
	L17	QD1	K28	QD		
	V24	QG1				
➤➤	L17	QD1	M35	QG	5.5	3.93
	V24	QG1				
➤	L17	QD1	I32	QG1	5.5	3.36
➤			L34	QD2		
	V24	QG2	I32	QG1		
			L34	QD2		
➤	L17	QD1	I32	QG2	5.5	4.77
			I41	QG2		
	V24	QG2	I32	QG2		
			I41	QG2		
	L17	HG	I32	QD1		
➤			I32	QD1	5.5	2.77
			I41	QD1		
➤	L17	HG	L34	QB	5.5	5.44
➤	L17	HG	L34	QD1	5.5	4.76
			V40	QG1		
➤➤	L17	HG	M35	QE	5.5	4.87
➤➤	L17	QD1	M35	QE	5.5	2.85
➤➤	L17	QD2	M35	QB	5.5	4.67
	V18	QG1	N27	QB		
➤	V24	QG1			5.5	4.89
➤	F19	QD	N27	QB	5.5	4.43
➤	F19	QD	N27	HA	5.5	5.53
			A42	HA		
➤	F19	QE	I32	QG1	5.5	3.93
			L34	QD2		
			R5	QG		
➤	F19	QE	I31	QD1		
➤			I32	QD1	5.5	3.25
			I41	QD1		
➤	F19	QE	A30	HA	5.5	2.62
			H6	HA		
➤	F19	HZ	I32	HB	5.5	4.56
➤	F19	HZ	A30	HA	5.5	3.00
			H6	HA		
➤	F19	HZ	A30	QB	5.5	3.79
			V36	QG2		
➤	F19	HZ	I31	QG1	5.5	3.98
			I32	QG1		
			L34	QD2		
➤	N27	QB	A30	HA	5.5	4.72
			H6	HA		
	N27	QB	I32	QG2		
➤			I41	QG2	5.5	5.45
➤	K28	QD	I41	QG2	5.5	2.83

	K28	QG	L28	QG		
			I32	HA		
➤			I41	HA	5.5	5.4
	K28	QG	I31	QD1		
			I32	QD1		
➤			I41	QD1	5.5	5.19
	K28	QE	I32	QG2		
➤			I41	QG2	5.5	4.28
	K28	QD	I31	QD1		
➤			I41	QD1	5.5	5.50
➤	I31	HB	V36	HB	5.5	5.50
			Q15	QG		
➤	I31	QG2	V36	QG1	5.5	4.48

DARR	ω1		ω2		UPL	d
➤	L17	CG	L34	CB	7.0	6.59
➤	N27	CA	A30	CA	7.0	5.76
	A42	CA				
➤	K28	CD	I41	CB	7.0	5.86
➤	K28	CD	A42	C	7.0	3.68
	G29	C	I32	CG2		
➤			I41	CG2	7.0	4.78
➤	G29	CA	I41	CB	7.0	3.73
➤	I31	CB	V36	CG1	7.0	6.41
➤➤	Q15	CD	M35	CE	7.0	4.5
➤➤	L17	CD1	M35	CE	7.0	4.95
	V24	CG2				
	L17	CD1	N27	CB		
➤	V24	CG2			7.0	5.84
➤	L17	CG	I32	CG1	7.0	4.20
➤			L34	CD2		
➤	L17	CG	I32	CG2	7.0	5.45
			I41	CG2		
➤	L17	CG	L34	CB	7.0	6.53
	L17	CD1	I31	CD1		
➤			I32	CD1	7.0	5.19
			I42	CD1		
	V24	CG2	I31	CD1		
			I32	CD1		
			I42	CD1		
➤	L17	CD1	I32	CG2	7.0	6.70
			I41	CG2		
	V24	CG2	I32	CG1		
			I41	CG1		
➤	F19	CE	G29	CA	7.0	6.46
➤	F19	CZ	G29	CA	7.0	7.03

➤	F19	CE	A30	CA	7.0	3.44
			H6	CA		
➤	F19	CZ	A30	CB	7.0	4.07
			V36	CG2		
➤	F19	CZ	A30	CA	7.0	3.87
			H6	CA		
	F19	CE	I31	CG2		
➤			I32	CG2	7.0	4.14
➤	F19	CE	I31	CG1		
➤			I32	CG1	7.0	4.37
			L34	CD2		
➤	F19	CD1	I31	CB	7.0	6.43
➤	F19	CE	I31	CB	7.0	5.36
➤	F19	CZ	I31	CB	7.0	5.76
	F19	CZ	I31	CG2		
➤			I32	CG2	7.0	5.22
➤	F19	CZ	I31	CG1	7.0	5.18
➤			I32	CG1		
			L34	CD2		
➤	F20	CE	I31	CG2		
➤			I32	CG2	7.0	7.02
	F4	CE	I31	CG2		
			I32	CG2		
	F20	CA	L17	CD1		
➤			V24	CG2	7.0	5.67
	S8	CA	L17	CD1		
			V24	CG2		
➤	N27	CB	A30	CA	7.0	5.36
			H6	CA		
➤	K28	CD	I41	CG2	7.0	4.57
➤	K28	CG	A42	C	7.0	3.85
➤	K28	CE	I41	CG2	7.0	5.84
➤	G29	CA	A42	C	7.0	6.98
	G38	CA				
➤	V36	CB	I31	CD1	7.0	5.65
			I32	CD1		
			I41	CD1		
➤	G33	CA	V36	CG1	7.0	5.89
	N15	QG	I31	QD1		
			I41	QD1		
➤	V35	QG	I31	QD1	7.0	3.86
			I41	QD1		

Table S3. Restraint and structure statistics.

Quantity	manual and automated analysis	manual analysis
Conformational restraints (per monomer):[a]		
Distance restraints from solid state NMR spectra:[a]	632	81
intraresidual	18	0
sequential ($\|i - j\| = 1$)	215	1
medium range ($2 \leq \|i - j\| \leq 4$)	202	21
long range ($\|i - j\| \geq 5$)	197	59
intramolecular	507	20
intra- or intermolecular	108	58
intermolecular	17	3
Restrained hydrogen bonds[b]	16	16
Dihedral angle restraints (ϕ/ψ)	38	38
Restraint violations:[c]		
CYANA target function value (Å^2)	1.90 ± 0.04	0.95 ± 0.34
RMS distance restraint violation (Å)	0.0023 ± 0.0005	0.0022 ± 0.0006
Maximal distance restraint violation (Å)	0.19	0.09
RMS dihedral angle restraint violation (°)	0.10 ± 0.08	0.018 ± 0.035
Maximal dihedral angle restraint violation (°)	1.99	1.01
RMSD to mean for the central 2 monomers:		
Backbone of residues 15–42 (Å)	0.89 ± 0.19	1.01 ± 0.16
All heavy atoms of residues 15–42 (Å)	1.14 ± 0.16	1.26 ± 0.14

[a]Each group of symmetrically equivalent distance restraints is counted as a single restraint. Distance restraints with multiple assignments are classified by the assignment spanning the shortest residue range.
[a]Each hydrogen bond was restrained by two upper and two lower distance bounds.
[c]Where applicable, the average value and the standard deviation over the 10 conformers that represent the NMR structure are given.

Table S4. Parameters used for solid-state NMR experiments for the structural calculation of the Aβ fibrils. DL: ^{13}C,^{15}N-abelled Aβ, diluted: labeling of Aβ in a ratio of ^{13}C,^{15}N:^{12}C,^{14}N = 1:3. Squared brackets indicate the values for the respective labeling (depicted also in brackets) if they are different from the other values.

Experiment	**DARR 20ms (DL)**	**DARR 50ms (DL)**	**DARR 200ms (DL)**	**DARR 400ms (DL)**	**DARR 400ms (diluted)**	**400us CHHC (DL)**	**400us CHHC (diluted)**
MAS frequency [kHz]	19	17	15	15	15	15	15
pulse ^{1}H power/dB – 100kHz	0.5	0.5	1.1	1.2	0.9	1	1
100							
pulse ^{13}C power/dB – 62.5kHz	-0.6	-0.6	-0.6	-0.6	0	-0.5	-0.4
62.5							
pulse ^{15}N power/dB - 50kHz			-0.6				
50							
Transfer 1	HC-CP	HC-CP	HC-CP	HC-CP	HC-CP	HC-CP	HC-CP
field [kHz] -^{1}H	74.9	74.9	80.3	81.2	78.5	79.4	79.4
field [kHz] -X	57.6	61.8	63.2	63.2	67.8	68.6	67.8
shape	tangent	tangent	tangent	tangent	tangent	tangent	tangent
carrier [ppm]	-	-	-	-	-	CA	CA
time [ms]	0.9	0.9	0.9	0.9	0.9	0.5	0.5
Transfer 2	DARR	DARR	DARR	DARR	DARR	H-H mixing	H-H mixing
field [kHz] -^{1}H	18.7	17.7	17.9	18.1	18.5	-	-
field [kHz] -^{13}C	-	-	-	-	-	-	-
field [kHz] - ^{15}N	-	-	-	-	-	-	-
shape	-	-	-	-	-	-	-
carrier [ppm]	-	-	-	-	-	-	-
time [ms]	20	50	200	400	400	0.4	0.4
t_1 increments	2560	2560	2560	2048	2048	1856	1856
sweep width (t_1) [kHz]	100	100	100	100	100	100	100
max. acq time (t_1) [ms]	12.80	12.80	12.80	10.24	10.24	9.28	9.28
t_2 increments	3968	3072	3072	3024	2560	2816	2816
sweep width (t_2) [kHz]	100	100	100	100	100	100	100
max. acq time (t_2) [ms]	19.84	15.36	15.36	15.12	12.80	14.08	14.08
^{1}H Spinal64 Decoupling power [kHz]	90	90	90	90	90	90	90
interscan delay [s]	3	2.5	2	2	2	2.3	2.3
number of scans	8	8	64	91	152	96	240
total measurement time [h]	17.3	14.6	100.8	124.9	208.6	114.5	286.1

Table S5. Parameters used for solid-state NMR experiments for the structural calculation of the Aβ(1-42) fibrils. DL: $^{13}C,^{15}N$-labelled Aβ, diluted: labeling of Aβ in a ratio of $^{13}C,^{15}N$: $^{12}C,^{14}N$ = 1:3, mixed: ^{13}C and ^{15}N-labelled Aβ in a ratio of 1:1. Squared brackets indicate the values for the respective labeling (depicted also in brackets) if they are different from the other values.

Experiment	**8ms PAR (DL)**	**8ms PAR (diluted)**	**6ms PAIN (mixed)**	**6ms PAIN (DL)**	**NHHC (mixed)**	**TEDOR (mixed)**
MAS frequency [kHz]	15	15	17	17	17	12.5
pulse ^{1}H power/dB – 100kHz **100**	0.6	1.3	0.8	0.8	0.7	4
pulse ^{13}C power/dB – 62.5kHz **62.5**	-0.6	-0.8	-0.6	-0.6	-0.6	2
pulse ^{15}N power/dB - 50kHz **50**	-1	-1.2	-0.65	-0.7	-0.7	-0.6
Transfer 1	HC-CP	HC-CP	HN-CP	HN-CP	HN-CP	HC-CP
field [kHz] -^{1}H	75.8	74.9	69.1	69.1	68.3	50.0
field [kHz] -X	65.5	61.8	55.0	55.0	56.3	40.2
shape	tangent	tangent	tangent	tangent	tangent	tangent
carrier [ppm]	-	-	-	-	-	-
time [ms]	0.9	0.9	1.2	1.2	1.2	0.9
Transfer 2	PAR	PAR	PAIN	PAIN	H-H mixing	TEDOR
field [kHz] -^{1}H	53.6	51.8	38.8	38.8	-	-
field [kHz] -^{13}C	52.0	52.6	36.7	36.7	-	50.0
field [kHz] - ^{15}N			35.7	35.7	-	*50.0*
shape	60	60	-	-	-	-
carrier [ppm]	-	-	-	-	-	55
time [ms]	8	8	6	6	0.5	10.8
t_1 increments	1856	1856	768	768	768	256
sweep width (t_1) [kHz]	100	100	50	50	50	12.5
max. acq time (t_1) [ms]	9.28	9.28	7.68	7.68	7.68	10.24
t_2 increments	2816	2816	2560	2560	2560	1536
sweep width (t_2) [kHz]	100	100	100	100	100	50
max. acq time (t_2) [ms]	14.08	14.08	12.80	12.80	12.80	15.36
^{1}H Spinal64 Decoupling power [kHz]	90	90	90	90	90	90
interscan delay [s]	2.5	2.5	2.8	2.8	2.2	2.5
number of scans	80	168	480	32	480	1024
total measurement time [h]	104.0	218.3	288.5	19.2	225.3	184.0

Glossary on solid-state NMR experiments

NCA(NCO): In this experiment magnetization is transferred from proton to nitrogen and further selectively to bound $^{13}C\alpha$ (^{13}CO). This leads to a 2D ^{13}C-^{15}N spectrum, where each peak represents a carbon attached to the respective nitrogen.

DARR (Dipolar **A**ssisted **R**otational **R**esonance): Is an experiment, where the magnetization is transferred from a proton to ^{13}C nuclei and further, assisted by the presence of protons, to other ^{13}C nuclei close in space. The longer the mixing time, the further away the magnetization is transferred. This leads to a two dimensional ^{13}C-^{13}C spectrum.

CHHC: Is a homonuclear experiment and leads to a 2D ^{13}C-^{13}C spectrum. During the experiment the magnetization is transferred from a carbon to a bound proton and then further transferred to other protons close in space. Finally, it is back-transferred from the proton to bound ^{13}C nuclei. Therefore, this experiment contains information about proton-proton distances.

PAR (^{13}C - ^{13}C **P**roton **A**ssisted **R**ecoupling): Same as DARR and CHHC, it is a homonuclear experiment leading to a 2D ^{13}C-^{13}C spectrum. However, a more specific transfer between the carbons is used compared to the DARR, exploiting cross terms between ^{13}C-^{1}H couplings. Consequently, it leads to less crowded spectra.

NHHC: Is a heteronuclear experiment, where the magnetization is transferred from a proton to nitrogen via cross polarization. It is transferred back to the proton, and further to other protons close in space, and finally back to the bound carbon for the detection. This leads to a 2D ^{13}C-^{15}N spectrum leading to distance information between protons.

PAIN (**P**roton **A**ssisted **I**nsensitive **N**uclei cross polarization): Same as NHHC, it is a heteronuclear experiment, where protons are involved in the polarization transfer. However, it uses a different mechanism of transfer of magnetization and therefore leads to different/additional information.

TEDOR (**T**ransferred **E**cho **DO**uble **R**esonance): Is a heteronuclear experiment, but in contrast to NHHC and PAIN the polarization transfer is achieved by direct recoupling of ^{15}N-^{13}C dipolar coupling. This leads to a 2D ^{13}C-^{15}N spectrum.

Glossary on solution-state NMR experiments

^{15}N-HMQC (**H**eteronuclear **M**ultiple-**Q**uantum **C**orrelation): Is a 2D ^{1}H-^{15}N experiment. Each peak represents a proton frequency in one dimension and in the other the frequency of the bound carbon. With this experiment, protons are connected to the respective carbon.

HNCA, HNCACB, HN(CO)CA: Are standard 3D assignment sequences. In the HNCA, magnetization is transferred from the proton to the bound nitrogen and further to the $^{13}C\alpha$ of the same and preceding amino acid residues. This leads to a 3D ^{1}H-^{15}N-^{13}C spectrum. The HNCACB starts the same way as the HNCA, but also the $^{13}C\beta$ of the same and preceding amino acid residues are detected. HNCA (and HNCACB) result in an ambiguous assignment of the amide resonance to both $^{13}C\alpha$ (and $^{13}C\beta$) nuclei. However, the HCN(CO)CA results only in the connection to the preceding amino acid residue leading to a unique assignment of the amide to the preceding residue and using the information provided form the HNCA also to the same amino acid residue.