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Optimized Precursor to Simplify Assignment Transfer between Backbone Resonances and Stereospecifically labelled Valine and Leucine Methyl Groups: Application to Human Hsp90 N-Terminal Domain

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Abstract:

Methyl moieties are highly valuable probes for quantitative NMR studies of large proteins. Hence, their assignment is of the utmost interest to obtain information on both interactions and dynamics of proteins in solution. Here, we present the synthesis of a new precursor that allows connection of leucine and valine pro-*S* methyl moieties to backbone atoms by linear ^{13}C-chains. This optimized ^{2}H/^{13}C-labelled acetolactate precursor can be combined with existing ^{13}C/^{2}H-alanine and isoleucine precursors in order to directly transfer backbone assignment to the corresponding methyl groups. Using this simple approach leucine and valine pro-*S* methyl groups can be assigned using a single sample without requiring correction of ^{1}H/^{2}H isotopic shifts on ^{13}C resonances. The approach was demonstrated on the N-terminal domain of human HSP90, for which complete assignment of Ala-β, Ile-δ_1, Leu-δ_2, Met-ε, Thr-γ and Val-γ_2 methyl groups was obtained.

Keywords: NMR, assignment, methyl groups, acetolactate, HSP90

Introduction:

Solution state NMR is the method of choice to characterize proteins at atomic level and to probe their dynamics over a wide range of biologically relevant timescales. However, for a long-time, study of high molecular weight proteins by NMR remained a challenge, notably due to the extensive line broadening of NMR signals in large proteins. Methyl groups have been widely studied and are extremely useful to overcome this issue that has hampered, in the past, quantitative NMR studies on large proteins. Indeed, due to the proton multiplicity and their favorable relaxation properties, methyl groups allow the detection of NMR signals even for large proteins (Tugarinov et al. 2003). Nowadays, methyl groups are important probes to investigate molecular dynamics (Sprangers and Kay 2007) and to provide functional insight (Rosenzweig et al. 2013; Mas et al. 2018) on assemblies weighing up to 1 MDa. Specific labelling of methyl groups on perdeuterated large proteins allows the measurement of long-range distance restraints, up to 12 Å. (Sounier et al. 2007; Ayala et al. 2020) and enables, in combination with other structural biology techniques such as SANS/SAXS (Lapinaite et al. 2013) or Cryo-EM (Gauto et al. 2019), to solve the structure of complexes of several hundreds of kDa.

For the past 20 years, a plethora of protocols overexpressing proteins in M9/$^{2}H_2O$ based *E.coli* growth medium and leading to specific protonation of methyl groups in perdeuterated proteins without scrambling of protons to other sites have been elaborated. On one hand, the direct incorporation of the methyl labelled amino acid in M9/$^{2}H_2O$ is employed for the specific labelling of $^{13}C^{1}H_3$-alanine (Isaacson et al. 2007; Ayala et al. 2009), $^{13}C^{1}H_3$-methionine (Gelis et al. 2007; Stoffregen et al. 2012) and $^{13}C^{1}H_3$-threonine (Velyvis et al. 2012; Ayala et al. 2020). On the other hand, as leucine, valine and isoleucine residues are at the end of irreversible metabolic pathways in *E.coli*, precursors can be incorporated in the growth medium for their cost-effective labelling. The first precursors introduced to label isoleucine or leucine and valine residues were 2-keto acids: α-ketobutyrate (Gardner et al. 1997) and α-ketoisovalerate (Goto et al. 1999; Hajduk et al. 2000; Gross et al. 2003), respectively. However, α-ketoisovalerate used as a precursor for leucine and valine residues is leading to a non-stereospecific labelling of both pro-*S* and pro-*R* $^{13}C^{1}H_3$ groups resulting in overcrowded spectra for high molecular weight proteins, even when both sensitivity and resolution have been improved using a non-stereospecific $^{13}C^{1}H_3/^{12}C^{2}H_3$ α-ketoisovalerate (Tugarinov and Kay 2004b).

To prevent peak overlaps and to facilitate studies of large molecular weight assemblies, methyl labelled acetolactate has been used as an alternative precursor (Gans et al. 2010). This

latter enables the stereospecific $^{13}C^{1}H_3$-labelling of valine and leucine methyl groups and therefore halves the number of peaks observed whilst improving by two the sensitivity of the spectrum as compared to labelling at 50% pro-*S* and 50% pro-*R* methyl moieties using optimized $^{13}C^{1}H_3/^{12}C^{2}H_3$ α-ketoisovalerate (Tugarinov and Kay 2004b). This interesting precursor also enhances the intensity of NOE cross peaks and increases the distance threshold at which NOE cross peaks can be detected by 20 % (Gans et al. 2010).

However, despite the tremendous progress made in protocols to selectively introduce protonated methyl groups in perdeuterated proteins, sequence specific assignment, essential for analyzing a variety of NMR data, remains an important challenge for large molecular assemblies. Several methods to solve the bottleneck of assignment of large proteins have been developed including parallel mutagenesis strategies (Amero et al. 2011), structure based approaches using the analysis of NOE cross peaks with various programs (Pritišanac et al. 2020), MAP-XSII (Xu and Matthews 2013), FLAMEnGO 2.0 (Chao et al. 2014), MAGMA (Pritišanac et al. 2017), MAGIC (Monneau et al. 2017), MethylFLYA (Pritišanac et al. 2019), MAUS (Nerli et al. 2021) or the "divide-and-conquer" approach (Gelis et al. 2007; Sprangers and Kay 2007) which is based on separation of large proteins into smaller fragments, assigning these and transferring the assignment back to the full-length protein. For proteins of moderate molecular weight or fragments of large assemblies for which backbone assignment is available, it is possible to connect methyl resonances to those of the backbone. This requires a sample with $^{13}C^{1}H_3$ labelled methyl groups connected to the backbone by a linear ^{13}C-chain. With such a sample, transfer from the assigned backbone to the methyl groups can be achieved using either unidirectional transfer from methyl groups to HN using (HM)CM(CGCBCA)NH experiments (Tugarinov and Kay 2003) or 'out and back' HCC relay triple resonance experiments (Tugarinov and Kay 2003; Ayala et al. 2012; Mas et al. 2013).

A combination of these techniques to assign methyl groups in addition with a stereospecific labelling, enhancing the sensitivity of the spectrum by a factor two and significantly reducing signal overlap, should lead to straightforward leucine and valine methyl group assignment. Labelling schemes, connecting non-stereospecifically both leucine and valine methyl groups (Tugarinov and Kay 2003), or only pro-*R* methyl moieties (Mas et al. 2013; Kerfah et al. 2015a), to the assigned backbone are already available. However, with such precursors additional samples are required either to stereospecifically assign the methyl group (Tugarinov and Kay 2004a; Gans et al. 2010) or to link the pro-*S* methyl to the pro-*R* one (Mas et al. 2013; Kerfah et al. 2015a). Here we introduce the synthesis of a new dissymmetric $^{13}C/^{2}H$-labelled acetolactate, a precursor that allows to directly connect the assigned leucine and valine

backbone atoms to the pro-*S* methyl groups via a linear ^{13}C chain using only one sample. This new labelling scheme has been applied to the N-terminal domain of human HSP90 (HSP90-NTD) and we present here the full assignment of methyl moieties of this protein.

Materials and Methods

Synthesis of 1, 2, 3-[$^{13}C_3$]- 4, 4, 4-[$^{2}H_3$]-acetolactate.

a) Synthesis of ethyl 1, 2, 3-[$^{13}C_3$]-3-oxo-butanoate. A solution of LiHMDS (7.80 g, 46.6 mmoles, 2.1 equiv.) in freshly dried THF (150 mL) was cooled to -78°C under argon. 1, 2-[$^{13}C_2$]-ethyl acetate (2.00 g, 22.2 mmoles, Cambridge Isotope Laboratory, CIL) was added dropwise. The resulting solution was stirred at -78°C for 15 min, then 1-[^{13}C]-acetyl chloride (1.60 mL, 22.2 mmoles, 1 equiv., CIL) was added dropwise. The resulting mixture was stirred at -78 °C for additional 30 min then quenched by addition of a 20% aqueous solution of ^{1}HCl (15 mL). After 3 extractions with Et_2O, the organics were combined, washed with saturated $Na^{1}HCO_3$ solution then dried over Na_2SO_4. Concentration under vacuum affords the desired product (2.88g) which was used in the next step without further purification).

^{1}H NMR:($C^{2}HCl_3$), δ: 4.21 (dq, O-CH_2, $^{3}J(^{1}H\text{-}^{1}H)$ = 7.1 Hz, $^{3}J(^{1}H\text{-}^{13}C)$ = 3.2 Hz, 2H); 3.46 (dt, $^{13}C\text{-}^{13}CH_2\text{-}^{13}C$, $^{2}J(^{1}H\text{-}^{13}C)$ = 6.5 Hz, $^{1}J(^{1}H\text{-}^{13}C)$ = 130.1 Hz, 2H); 2.28 (dd, $CH_3\text{-}^{13}C$, $^{3}J(^{1}H\text{-}^{13}C)$ = 1.4 Hz, $^{2}J(^{1}H\text{-}^{13}C)$ = 6.1 Hz, 3H), 1.30 (t, OCH2-CH3, $^{3}J(^{1}H\text{-}^{1}H)$ = 7.1 Hz, 3H).

b) Synthesis of ethyl 1, 2, 3-[$^{13}C_3$]-2-[$^{13}C^{1}H_3$]-3-oxo-butanoate. $^{13}C^{1}H_3$-I (752 mL, 11.99 µmoles, 1.1 equiv, CIL) was slowly added to a solution of ethyl 1, 2, 3-[$^{13}C_3$]-3-oxo butanoate (1.45 g, 10.90 mmoles) in EtO^{1}H (50 mL) cooled to 0°C before addition of K_2CO_3 (1.66 g, 11.99 mmoles, 1.1 equiv.). The resulting suspension was warmed to room temperature then stirred for 18 h. The mixture was concentrated to the fifth before addition of a large volume of Et_2O. Excess of K_2CO_3 was filtered off and the filtrate concentrated under vacuum to the fifth before a further addition of Et_2O and a second filtration. Concentration under vacuum affords the desired product (1.03 g) as a colorless oil which was used in the next step without further purification.

^{1}H NMR:($C^{2}HCl_3$), δ: 4.21 (dq, O-CH_2, $^{3}J(^{1}H\text{-}^{1}H)$ = 7.1 Hz, $^{3}J(^{1}H\text{-}^{13}C)$ = 3.0 Hz, 2H); 3.50 (dm, $^{13}C\text{-}^{13}CH\text{-}^{13}C$, $^{1}J(^{1}H\text{-}^{13}C)$ = 129.0 Hz, 1H); 2.24 (dd, $CH_3\text{-}^{13}C$, $^{3}J(^{1}H\text{-}^{13}C)$ = 1.3 Hz, $^{2}J(^{1}H\text{-}^{13}C)$ = 6.0 Hz, 3H), 1.36 (dm, $^{13}CH_3$, $^{1}J(^{1}H\text{-}^{13}C)$ = 129.0 Hz, 3H), 1.28 (t, $OCH_2\text{-}CH_3$, $^{3}J(^{1}H\text{-}^{1}H)$ = 7.1 Hz, 3H).

c) Synthesis of ethyl 1, 2, 3-[$^{13}C_3$]-2-[$^{13}C^{1}H_3$]-2-[$O^{1}H$]-3-oxo-butanoate. To a solution of ethyl 1, 2, 3-[^{13}C3]-2-[$^{13}C^{1}H_3$]-3-oxo butanoate (995 mg, 6.72 mmoles) in DMSO (8 mL), Cs_2CO_3 was added (440 mg, 1.35 mmoles, 0.2 equiv.). After O_2 bubbling for 15 min., $P(OEt)_3$ (233 mL, 1.35 mmoles, 0.2 equiv.) was added. The resulting solution was stirred under O_2 atmosphere

for 20 h. A large volume of Et_2O was then added followed by a saturated NaCl solution. The resulting phases were separated and the aqueous one was extracted one more time with Et_2O. The organics were combined, dried over Na_2SO_4 then concentrated under vacuum to obtain ethyl 1, 2, 3-[$^{13}C_3$], 2-[$^{13}C^1H_3$], 2-[O^1H]-3-oxobutanoate as a yellow oil (1.142 g) and pure enough to be used in the next step without further purification.

^1H NMR:(C^2HCl_3), δ: 4.25 (dq, O-CH_2, $^3J(^1H\text{-}^1H)$ = 7.1 Hz, $^3J(^1H\text{-}^{13}C)$ = 3.2 Hz, 2H); 4.17-4.24 (m, OH, 1H), 2.27 (dd, CH_3-^{13}C, $^3J(^1H\text{-}^{13}C)$ = 1.1 Hz, $^2J(^1H\text{-}^{13}C)$ = 6.1 Hz, 3H), 1.36 (dm, $^{13}CH_3$, $^1J(^1H\text{-}^{13}C)$ = 134.2 Hz, 3H), 1.28 (t, OCH2-CH3, $^3J(^1H\text{-}^1H)$ = 7.1 Hz, 3H).

d) Synthesis of sodium 1, 2, 3-[$^{13}C_3$]-2-[$^{13}C^1H_3$]-2-[O^2H]-3-oxo-4, 4, 4-[$^2H_3$]-butanoate. To a solution of ethyl 1, 2, 3-[$^{13}C_3$]- 2-[$^{13}C^1H_3$]-2-[O^1H]-3-oxobutanoate (1.09 g) in ^2H_2O (4 mL), 0.4 equivalents of a solution of NaO^2H (2.5 M) in ^2H_2O were added dropwise in 40 min, using a syringe pump under argon. As soon as the addition was completed, ^1H NMR was carried out on a sample (few µL) in ^2H_2O in order to calculate the conversion percentage (*ratio* between the amount of hydrolyzed product (quadruplet at 1.60 ppm) and the amount of starting material (quadruplet at 1.7 ppm)). 1.1 equivalent of NaO^2H solution (2.5 M) was added over 30 min with the syringe pump. As soon as the addition was completed, an extraction with diethyl ether was carried out in order to remove the by-product coming from the previous step whose NMR signals prevent a good follow-up of the hydrogen/deuterium ($^1H/^2H$) exchange on the 4-CH_3 (2.2 ppm). The $^1H/^2H$ exchange on 4-CH_3 was then monitored by ^1H NMR and carried out by successive addition of NaO^2H (2.5 M) until the integral of the doublet corresponding to the CH_3 reaches the value of 0.1 when the quadruplet at 1.6 ppm integrates for 1.5. The reaction was immediately neutralized with a concentrated ^2HCl solution to neutral pH and then buffered with Tris-^1HCl, (1.0 M, pH 7.5 in ^2H_2O). The concentration of the resulting solution was then determined by ^1H NMR using methanol or acetonitrile as internal reference. The final product (3.02 mmoles) was stored at -80°C.

^1H NMR:(C^2HCl_3), δ: 1.37 (dq, $^{13}CH_3$, $^1J(^1H\text{-}^{13}C)$ = 129.3 Hz, $^2J(^1H\text{-}^{13}C)$ = 3.9 Hz, 1H).

Preparation of isotopically labelled HSP90-NTD samples.

E. coli BL21-DE3-RIL cells transformed with a pET-28 plasmid encoding the N-Terminal domain of HSP90 α from *Homo Sapiens* (HSP90-NTD) with a His-Tag and a TEV cleavage site were progressively adapted in three stages over 24 h to M9/^2H_2O. In the final culture, bacteria were grown at 37°C in M9 medium with 99.85 % ^2H_2O (Eurisotop), 1 g/L $^{15}N^1H_4Cl$

(Sigma Aldrich) and 2 g/L D-glucose-d_7 (for U-[^{2}H, ^{12}C, ^{15}N] HSP90-NTD samples) or D-glucose-$^{13}C_6$-d_7 (CIL) (for U-[^{2}H, ^{13}C, ^{15}N] HSP90-NTD samples).

For methyl specifically labelled samples, the methyl labelled precursors or amino-acids were added to the media when the O.D at 600 nm reached 0.6 (Kerfah et al. 2015c):

- Labelling scheme (A): for production of the U-[^{2}H, ^{15}N, ^{13}C], Ile-[2, 3, 4, 4-$^2H_4$; 1, 2, 3, 4-$^{13}C_4$; $^{13}C^1H_3]^{\delta1}$/[$^{12}C^2H_3]^{\gamma2}$], Leu-[2, 3, 3, 4-$^2H_4$; 1, 2, 3, 4-$^{13}C_4$; [$^{13}C^1H_3]^{pro\text{-}S}$/[$^{12}C^2H_3]^{pro\text{-}R}$], Val-[2, 3-$^2H_2$; 1, 2, 3-$^{13}C_3$; [$^{13}C^1H_3]^{pro\text{-}S}$/[$^{12}C^2H_3]^{pro\text{-}R}$] HSP90-NTD, a solution containing the sodium 1, 2, 3-[$^{13}C_3$]-2-[$^{13}C^1H_3$]-2-[O^2H]-3-oxo-4, 4, 4-[$^2H_3$]-butanoate precursor was added at a concentration of 172 mg/L 1 h before induction. 40 min later (20 minutes before induction) a solution containing 60 mg/L of sodium (*S*)-2-hydroxy-2-(1',1'-[$^2H_2$], 1', 2'-[$^{13}C_2$])ethyl-3-oxo-1,2,3-[$^{13}C_3$]-4,4,4-[$^2H_3$]butanoate (Kerfah et al. 2015a) was added to the medium.
- Labelling scheme (B): for production of the U-[^{2}H, ^{15}N, ^{12}C], Ala-[$^{13}C^1H_3]^{\beta}$, Met-[$^{13}C^1H_3]^{\varepsilon}$, Leu/Val-[$^{13}C^1H_3]^{pro\text{-}S}$, Ile-[$^{13}C^1H_3]^{\delta1}$, Thr-[$^{13}C^1H_3]^{\gamma}$ HSP90-NTD, a HLAM-$A^{\beta}I^{\delta1}M^{\varepsilon}LV^{proS}T^{\gamma}$ kit, purchased from NMR-Bio, was added before induction according to the manufacturer's protocol.
- Labelling scheme (C): U-[^{2}H, ^{15}N, ^{12}C] samples labelled on a single type of methyl group were produced in small scales (21 mL) to identify A^{β}, M^{ε}, T^{γ} methyl type (3 samples) or to complete assignment using single point mutants (Amero et al. 2011) (33 samples, list of mutants presented in the legend of Fig. S4). Single point amino acid mutations were generated by GeneCust. For each of these samples a single type of methyl groups was labelled by addition of the corresponding NMR-Bio kit (SLAM-A^{β}, SLAM-M^{ε}, SLAM-$I^{\delta1}$, SLAM-T^{γ} or DLAM-LV^{proS}) in M9/^2H_2O media 1 h before induction.

Protein production was induced by the addition of IPTG to a final concentration of 0.5 mM. The cultures were grown overnight at 20°C before harvesting. Cells were collected by centrifugation at 5500 g for 20 min at 4°C then lysed by sonication on ice in a buffer containing 20 mM phosphate sodium buffer at pH 7.4, 0.5 M NaCl, 0.05% β-ME, antiprotease (cOmplete® EDTA free, 1 tablet for 50 mL), 50 µg/mL DNAse (Sigma Aldrich), 50µg/mL RNAse (Euromedex), and 0.25 mg/mL Lysozyme (Euromedex). After removal of cell debris by centrifugation (45,000×g, 30 min, 4 °C), the supernatant was purified using an affinity chromatography step (Ni-NTA, Superflow, QIAGEN) (labelling scheme A, B and C), followed by a size exclusion chromatography step (16/600 Superdex 75 PG, GE Healthcare) (labelling

schemes A and B only). The gel filtration column was run with an isocratic step of the NMR buffer (20 mM Hepes, 150 mM NaCl, 1 mM TCEP, pH 7.5).

The HSP90-NTD proteins were concentrated, using an Amicon® 4 Centrifugal Filter Unit with a 10,000 MWCO (Merck), either in a 90%/10% $^{1}H_2O/^{2}H_2O$ or in a 100% $^{2}H_2O$ buffer containing 20 mM Hepes, 150 mM NaCl, 1 mM TCEP, pH 7.5. For labelling schemes A and B, samples were concentrated to 0.5 mM and 200 µL of each sample was loaded in 4 mm shigemi tube. The wild type and single point mutants of HSP90-NTD proteins labelled on only one methyl type (labelling scheme C) were concentrated at [0.1-0.4] mM and 40 µL of each sample was loaded in a 1.7 mm NMR tube.

NMR Spectroscopy

All NMR experiments acquired on HSP90-NTD samples were recorded at 298 K. 2D ^{1}H-^{13}C SOFAST methyl TROSY (Amero et al. 2009) experiments to identify each methyl type as well as to assign individual methyl signals using single point mutants were recorded for an average duration of ~ 1.5 h each, on a spectrometer operating at a ^{1}H frequency of 850 MHz and equipped with a 1.7 mm cryogenically cooled, pulsed-field-gradient triple-resonance probe. All other NMR experiments were acquired using Bruker Avance III HD spectrometers equipped with 5 mm cryogenic probes (operating at a ^{1}H frequency of 600 or 950 MHz).

The 3D HCC, HC(C)C and HC(CC)C experiments (Tugarinov and Kay 2003; Ayala et al. 2009, 2012; Mas et al. 2013) were acquired on a spectrometer operating at a ^{1}H frequency of 600 MHz for a total duration of 4 days, using a 0.5 mM sample of U-[^{2}H, ^{15}N, ^{13}C], Ile-[2, 3, 4, 4-$^{2}H_4$; 1, 2, 3, 4-$^{13}C_4$; $^{13}C^{1}H_3]^{\delta1}/[^{12}C^{2}H_3]^{\gamma2}$], Leu-[2, 3, 3, 4-$^{2}H_4$; 1, 2, 3, 4-$^{13}C_4$; $[^{13}C^{1}H_3]^{pro\text{-}S}/[^{12}C^{2}H_3]^{pro\text{-}R}$], Val-[2, 3-$^{2}H_2$; 1, 2, 3-$^{13}C_3$; $[^{13}C^{1}H_3]^{pro\text{-}S}/[^{12}C^{2}H_3]^{pro\text{-}R}$] HSP90-NTD. The interscan delay was adjusted to 0.5-0.6 s, the heteronuclear ^{1}H -> ^{13}C transfer delay was set to 4 ms ($1/(2 \times {}^{1}J_{HC})$) and the homonuclear ^{13}C -> ^{13}C transfer delay was fixed to 12.5 ms. The acquisition times were adjusted to 8-10.7 ms in the ^{13}C indirect dimension, and to 70 ms in ^{1}H direct dimension.

For the sequential assignment of backbone resonances, a set of 6 BEST-TROSY 3D triple resonance experiments (HNCA, HN(CA)CB, HNCO, HN(CA)CO, HN(CO)CA and HN(COCA)CB (Favier and Brutscher 2019) were acquired on a Bruker Avance III HD spectrometer equipped with a cryogenic probe and operating at a ^{1}H frequency of 600 MHz for a total duration of 11 days using a 0.5 mM sample of the U-[^{2}H, ^{15}N, ^{13}C] HSP90-NTD.

The 3D CCH HMQC-NOESY-HMQC NMR experiment (Tugarinov et al. 2005; Törner et al. 2020) was recorded over 3 days on a spectrometer operating at a ^{1}H frequency of 950 MHz

using a 0.5 mM sample of U-[^{2}H, ^{15}N, ^{12}C], Ala-[^{13}C^{1}H$_3$]$^{\beta}$, Met-[^{13}C^{1}H$_3$]$^{\varepsilon}$, Leu/Val-[^{13}C^{1}H$_3$]$^{pro\text{-}S}$, Ile-[^{13}C^{1}H$_3$]$^{\delta 1}$, Thr-[^{13}C^{1}H$_3$]$^{\gamma}$ HSP90-NTD. The interscan delay was set to 1.1s. The heteronuclear ^{1}H -> ^{13}C transfer delay was set to 4 ms (1/(2 x $^{1}J_{HC}$)). The acquisition times in the ^{13}C indirect dimension were set to 24.6 ms, (t_{1max}) and to 18.6 ms (t_{2max}). In the ^{1}H direct dimension t_{3max} was fixed to 80 ms. The NOE mixing period was set to 500 ms to detect a maximum number of long-range intermethyl NOEs.

Data processing and analysis

All data were processed and analyzed using nmrPipe/nmrDraw (Delaglio et al. 1995) and CcpNMR (Vranken et al. 2005). Automated methyl assignment was performed using MAGIC software (Monneau et al. 2017) using the reference structure of HSP90-NTD (PDB: 1YES). Input NOE lists for MAGIC were created with CcpNMR. MAGIC was run with a score threshold factor of 1 and distance thresholds of 7–10 Å using all inter methyl NOEs detected (S/N threshold of 5 was used) and given the assignment of isoleucines, leucines and valines previously obtained by the three ‘out and back’ HCC experiments as well as alanine, methionine and threonine methyl groups assigned by mutagenesis as additional input.

Results and Discussion

To assign methyl groups of large perdeuterated proteins, previously assigned backbone resonances of these proteins can be used (Tugarinov and Kay 2003; Ayala et al. 2012; Mas et al. 2013). Nonetheless, to do so, methyl groups need to be connected, via a linear chain of ^{13}C, to the backbone atoms in order to be able to apply optimized experiments to high molecular weight proteins. Strategies to label stereospecifically leucine and valine pro-*R* methyl groups and to connect them to backbone nuclei have already been proposed (Mas et al. 2013). However, it has to be noted that pro-*S* methyl groups are often chosen over pro-*R* methyl groups as they are both easier and cheaper to label stereospecifically (Gans et al. 2010). In order to simplify assignment of pro-*S* methyl groups using already assigned backbone resonances and to avoid the need of an additional sample to link the pro-*S* methyl to the pro-*R* one, a sample connecting the pro-*S* $^{13}C^{1}H_3$-methyl groups to the backbone atoms by a linear ^{13}C-chain and labelled with $^{12}C^{2}H_3$ on pro-*R* methyl moieties to avoid signal loss would be optimal.

Synthesis of optimally labelled acetolactate precursors and proteins.

Taking into account the specificity of leucine/valine metabolic pathway in *E. coli*, such an optimal labelling scheme can be achieved in M9/$^{2}H_2O$ medium using $^{13}C/^{2}H$ glucose (Kerfah et al. 2015c) as a carbon source together with 1, 2, 3-[$^{13}C_3$]-2-[$^{13}C^{1}H_3$]-2-[$O^{2}H$]-3-oxo-4, 4, 4-[$^{2}H_3$]-butanoate as suitably labelled acetolactate precursor. However, this latter cannot be synthetized from commercially available materials by the traditional route starting from acetoacetate (Gans et al. 2010) since the corresponding labelled starting material is not commercially available. Indeed, acetolactate chemical synthesis is achieved by reaction of iodomethane on acetoacetate (Gans et al. 2010). Whilst both $^{13}C^{1}H_3$ labelling of the methyl substituent in position 2 and deuteration of the methyl group in position 4 can be obtained using $^{13}C^{1}H_3I$ as a starting synthesis material and hydrogen/deuterium exchange in controlled basic conditions (Gans et al. 2010), respectively, the ^{13}C labelling of only the first three carbons of the main chain using commercially available labelled acetoacetate materials is not achievable. Therefore, as acetoacetate can be obtained by condensation of two acetate moieties (Epstein J et al. 1977), we decided to set up a synthesis of dissymmetrically labelled acetoacetate starting from commercially available ethyl 1, 2-[$^{13}C_2$]- acetate with 1-[^{13}C]-acetyl chloride. Based on reported procedures, we established a 4-step synthesis (Fig. 1) allowing to prepare the desired precursor with an overall yield of 27%. In brief, the dissymmetry is achieved by the Claisen condensation of ethyl-1,2,[$^{13}C_2$]-acetate with 1-[^{13}C]-acetyl chloride using an optimization of a

reported procedure (Epstein J et al. 1977) (a), followed by an alkylation in position 2 using $^{13}C^{1}H_3I$ (b) and a subsequent hydroxylation in position 2 (c). Finally, the last step combining both saponification of the ester and a hydrogen/deuterium exchange in position 4 is performed under controlled basic conditions (d). This last step is very delicate and requires a fine control of the basic condition as a methyl rearrangement above a pH of 13.5 can take place resulting in the interconversion of both methyl groups (Gans et al. 2010). Steps (b) and (c) are not stereoselective, hence, products of these latter steps were produced as racemic mixtures. The optimally labelled acetolactate, unstable at room temperature, was aliquoted and stored at -80°C.

The synthetized acetolactate precursor was incorporated in *E. coli.* M9/$^{2}H_2O$ culture media without any further purification steps to label the overexpressed protein. Frozen acetolactate vials were thawed right before addition in the culture medium to avoid degradation or methyl rearrangement. It has to be noted that only the 2-(*S*) stereoisomer of acetolactate is converted *in vivo* by ketol–acid reductoisomerase (EC1.1.1.86) and dihydroxyacid dehydratase (EC 4.2.1.9) to form the stereospecifically labelled 2-keto-isovalerate. This latter is afterwards directly converted into valine or combined with $^{13}C/^{2}H$-pyruvate, derived from the $^{13}C/^{2}H$-glucose, to produce leucine with the desired labelling pattern. The 2-(*R*) stereoisomer of acetolactate is, itself, not a substrate of ketol–acid reductoisomerase and hence, induces no scrambling (Gans et al. 2010). The synthetized 1, 2, 3-[$^{13}C_3$]-2-[$^{13}C^{1}H_3$]-2-[$O^{2}H$]-3-oxo-4, 4, 4-[$^{2}H_3$]-butanoate can be mixed with other known precursors allowing also to connect methyl groups, such as Ile-δ_1 (Kerfah et al. 2015b, a; Törner et al. 2020), Ile-γ_2 (Ayala et al. 2012) or Ala-β (Ayala et al. 2009; Kerfah et al. 2015a; Törner et al. 2020), to backbone nuclei using a linear ^{13}C chain. In this study, we chose to label our sample on both leucine/valine pro-*S* and isoleucine-δ_1 methyl moieties, by adding Ile-δ_1 precursor (sodium (S)-2-hydroxy-2-(1',1'-[$^{2}H_2$], 1', 2'-[$^{13}C_2$])ethyl-3-oxo-1,2,3-[$^{13}C_3$]-4,4,4-[$^{2}H_3$]-butanoate – (Kerfah et al. 2015a,b)) together with our new optimized acetolactate. The isoleucine precursor was added in the culture medium 40 min later than the new precursor in order to take into account co-incorporation incompatibilities between both the leucine/valine precursor and the isoleucine one. Indeed, enzymes from ILV-pathway have a tendency to process preferentially isoleucine precursor instead of leucine/valine precursors (Kerfah et al. 2015b, c). Incorporation of both precursors during the protein expression did not lead to a significantly different HSP90-NTD yield with regards to the yields obtained in standard M9/$^{2}H_2O$ media. No scrambling was detected neither to the pro-*R* methyls groups of leucine and valine nor to the isoleucine-γ_2 site (Fig. S1a).

Connection of $I^{\delta1}L^{\delta2}V^{\gamma2}$ methyl groups to C_α and C_β atoms.

Using this new precursor, it is possible to directly correlate pro-*S* methyl groups of leucine and valine residues to their respective C_α and C_β. We decided to apply this strategy to the N-terminal domain of human HSP90 (HSP90-NTD), an extensively studied protein, whose isoform assignment (α and β), including partial assignment of its methyl groups, is available (Jacobs et al. 2006; Elif Karagöz et al. 2011; Park et al. 2011; Lescanne et al. 2017, 2018). Here, we have focused on the N-terminal domain of the α isoform, a 29 kDa protein that contains 20 isoleucine, 18 leucine and 11 valine residues. This dynamic protein is particularly challenging to assign using automatic methyl assignment methods and reported success rates are ranging from 27 % to 69 % (Pritišanac et al. 2017; Monneau et al. 2017; Pritišanac et al. 2019, 2020). In our hands, only 34 % of the methyl groups (30/87) could be assigned automatically with 1) a single assignment, 2) a high NOE assignment completeness of the strip related to each peak (> 50 %) and 3) a high total confidence score value (≥ 7) (Table S1) using the HSP90-NTD X-ray structure (PDB: 1YES) and experimentally detected NOE network. Therefore, this protein is a good candidate to assess our experimental strategy based on new precursors.

To do so, 200 µL at 0.5 mM of an optimally labelled sample was used to acquire three 'out and back' HCC, HC(C)C and HC(CC)C experiments (Tugarinov and Kay 2003; Ayala et al. 2009, 2012; Mas et al. 2013) connecting labelled $I^{\delta1}L^{\delta2}V^{\gamma2}$ methyls groups to $I^{\gamma1}L^{\gamma}V^{\beta}$, $I^{\beta}L^{\beta}V^{\alpha}$ and $I^{\alpha}L^{\alpha}$ resonances. 100 % and 94 % of the expected C_β and C_α coherences, respectively, were observed for the 29 kDa HSP90-NTD at 298 K (τ_C *c.a.* 20 ns) (Fig. 2). The three missing C_α resonances correspond to residues L56, I26 and I110, two of them being affected by extensive line broadening due to conformational exchange. With such a high percentage of observed C_α and C_β resonances we demonstrate the applicability of this method for medium size proteins. In order to validate the strategy for larger proteins, the labelling scheme was applied to the 87 kDa hetero hexameric protein prefoldin from Pyrococcus horikoshii, containing 2α and 4β-subunits. Only the β-subunits were labelled with the optimal labelling schemes described above, whilst the α-subunits remained perdeuterated (Fig. S1b). Three HCC experiments were collected on this 0.2 mM sample of prefoldin at 310 K (τ_C *c.a.* 60 ns). 95% and 60% of the expected C_β and C_α coherences, respectively, were observed (Fig. S2) despite the high molecular weight of the protein and the presence of doubled peaks due to the presence of two inequivalent β-subunits in the $\alpha_2\beta4$ hexameric prefoldin (Ohtaki et al. 2008). Such HCC experiments were also acquired at 343 K (τ_C *c.a.* 30 ns) on this hyperthermophilic prefoldin

sample, enabling transfer of assignment between backbone atoms and methyl groups (Törner et al. 2021).

Application to the sequence specific assignment of $I^{\delta1}L^{\delta2}V^{\gamma2}$ methyl groups of HSP90-NTD

Backbone sequential assignment was performed using 6 'BEST-TROSY' triple resonance experiments (Favier and Brutscher 2019). C_α and C_β resonances were assigned for 89 % and 80 % of the residues of HSP90-NTD respectively, excluding the loosely structured N-terminal [1-16] and C-terminal [225-236] regions. The segment L103-T115, that covers the ligand binding site, is invisible by NMR due to dynamics in the µs-ms timescale. Transfer of sequentially assigned backbones resonances to isoleucine-δ_1, leucine-δ_2 and valine-γ_2 methyl groups was achieved using 'out and back' HCC experiments acquired on a U-[^{2}H, ^{15}N, ^{13}C], Ile-[2, 3, 4, 4-$^{2}H_4$; 1, 2, 3, 4-$^{13}C_4$; $^{13}C^1H_3]^{\delta1}$/$[^{12}C^2H_3]^{\gamma2}$], Leu-[2, 3, 3, 4-$^{2}H_4$; 1, 2, 3, 4-$^{13}C_4$; $[^{13}C^1H_3]^{pro\text{-}S}$/$[^{12}C^2H_3]^{pro\text{-}R}$], Val-[2, 3-$^{2}H_2$; 1, 2, 3-$^{13}C_3$; $[^{13}C^1H_3]^{pro\text{-}S}$/$[^{12}C^2H_3]^{pro\text{-}R}$] labelled HSP90-NTD sample. 2D extracts from the HCC experiments were compared with the corresponding ones from the 3D HNCA and HN(CA)CB experiments and using C_α and C_β resonances, all the $I^{\delta1}L^{\delta2}V^{\gamma2}$ methyl groups could be unambiguously connected to previously assigned backbone atoms (Fig. 2). The assignment was transferred in one step, very simply and efficiently without having to correct for the isotopic shifts (Kerfah et al. 2015a). One must note that the methyl-δ_1 of Ile-33 and Ile-128 are superimposed in the 2D methyl-TROSY spectrum, but were unambiguously connected to the C_α resonances of both amino acids (Fig. S3). Remained unassigned only methyl groups of L103, I104, L107 and I110 for which backbone atoms are NMR-invisible due to extensive conformational exchange. Therefore, single point mutagenesis was used to assign three of these last four $I^{\delta1}$ or $L^{\delta2}$ resonances (I104, L107 and I110) (Fig. 3). The remaining residue, L103, was assigned by a careful re-analysis of both HCC and backbone triple resonance experiments performed after the assignment of I104.

Sequence specific assignment of A^{β} M^{ε} T^{γ} methyl groups of HSP90-NTD

In the previous U-[^{2}H, ^{15}N, ^{13}C], Ile-[2, 3, 4, 4-$^{2}H_4$; 1, 2, 3, 4-$^{13}C_4$; $^{13}C^1H_3]^{\delta1}$/$[^{12}C^2H_3]^{\gamma2}$], Leu-[2, 3, 3, 4-$^{2}H_4$; 1, 2, 3, 4-$^{13}C_4$; $[^{13}C^1H_3]^{pro\text{-}S}$/$[^{12}C^2H_3]^{pro\text{-}R}$], Val-[2, 3-$^{2}H_2$; 1, 2, 3-$^{13}C_3$; $[^{13}C^1H_3]^{pro\text{-}S}$/$[^{12}C^2H_3]^{pro\text{-}R}$] HSP90-NTD sample, only $I^{\delta1}L^{\delta2}V^{\gamma2}$ methyl groups were connected to backbone by a linear ^{13}C-chain. We did not incorporate labelled alanine in HSP90-NTD culture for the sample used to acquire the HCC experiments although it is commercially available with an optimal labelling pattern (1, 2, 3-$^{13}C_3$, 2-^{2}H-Ala). We recommend for future studies to incorporate labelled alanine in the culture medium to decrease the numbers of mutants

required to complete the assignment. Regarding methionine and threonine residues, their assignment cannot be undertaken using HCC experiments. Indeed, the sulfur atom present on methionine residues prevents the use of HCC experimens to assign methionine methyl moieties from available backbone assignment and the Thr-[1, 2, 3-^{13}C, 2, 3-^{2}H$_2$, ^{13}C^{1}H$_3$-γ] labelled amino acid is not commercially available impeding the use of HCC 3D experiments to transfer assignment from backbone to threonine methyl groups in large proteins. Therefore, in order to assign the remaining 38 A$^{\beta}$ M$^{\varepsilon}$ and T$^{\gamma}$ methyl groups, we decided to use a combination of both single point mutations and through space intermethyl NOE correlation peaks. To assign the remaining A$^{\beta}$, M$^{\varepsilon}$ and T$^{\gamma}$ methyl groups using inter-methyl NOE-connectivities a U-[^{2}H, ^{15}N, ^{12}C], Ala-[^{13}C^{1}H$_3$]$^{\beta}$, Met-[^{13}C^{1}H$_3$]$^{\varepsilon}$, Leu/Val-[^{13}C^{1}H$_3$]$^{pro\text{-}S}$, Ile-[^{13}C^{1}H$_3$]$^{\delta 1}$, Thr-[^{13}C^{1}H$_3$]$^{\gamma}$ HSP90-NTD sample was required. Indeed, intermethyl NOE connectivities between previously assigned isoleucine, leucine and valine residues and unassigned A$^{\beta}$, M$^{\varepsilon}$ and T$^{\gamma}$ methyl groups simplify the assignment of methionine, threonine and alanine methyl moieties.

First small-scale samples with only one type of methyl group labelled A$^{\beta}$, M$^{\varepsilon}$ or T$^{\gamma}$ were prepared to identify the amino acids type corresponding to each correlation remaining to assign in the 2D methyl-TROSY spectrum. Then, we overexpressed and purified in small scale 30 single point ^{13}C^{1}H$_3$-labelled mutants. In order to minimize secondary chemical shifts replacement amino acids that were structurally similar to the substituted amino acid were chosen (Crublet et al. 2014) (Fig. S4).

Each of these samples containing 100 to 500 µg of ^{13}C^{1}H$_3$-labelled single point mutant of HSP90-NTD were used to acquire a 2D SOFAST-methyl-TROSY spectrum (Amero et al 2009; Amero et al 2011) on a NMR spectrometer operating at a ^{1}H frequency of 850 MHz and equipped with a sample changer and a 1.7 mm cryogenic probe head. This mutant library allowed us to assign unambiguously 27 methyl groups. Three spectra of single point mutants were more complex to analyze due to chemical shift perturbations that result from the introduced mutation (Fig. S5). Out of the 38 A$^{\beta}$, M$^{\varepsilon}$ and T$^{\gamma}$ methyl groups, eleven remained unassigned after the first analysis of the mutant library, among them eight methyls (one M$^{\varepsilon}$, two A$^{\beta}$ and five T$^{\gamma}$ methyl groups) for which mutants were not available and three T$^{\gamma}$ methyl groups whose mutant spectra were challenging to analyze. The sole unassigned methionine methyl signal was unambiguously assigned as the last remaining methionine residue (M98).

To complete the assignment for the last 10 methyl groups, a 0.5 mM sample of U-[^{2}H, ^{15}N, ^{12}C], Ala-[^{13}C^{1}H$_3$]$^{\beta}$, Met-[^{13}C^{1}H$_3$]$^{\varepsilon}$, Leu/Val-[^{13}C^{1}H$_3$]^{proS}, Ile-[^{13}C^{1}H$_3$]$^{\delta 1}$, Thr-[^{13}C^{1}H$_3$]$^{\gamma}$ labelled HSP90-NTD was prepared to acquire a 3D HMQC-NOESY-HMQC. A total of 344 intermethyl NOEs cross peaks with a S/N ≥ 5 were detected between methyls distant by up to

10 Å. Examples of intermethyl NOE and a matrix presenting all the observed NOEs are displayed in Fig. 4. To assign the remaining 2 alanines and 8 threonines, the NOE connectivities and the previously assigned methyls (49 $I^{\delta 1}L^{\delta 2}V^{\gamma 2}$ and 28 A^{β} M^{ε} and T^{γ} methyl groups) were used as input for the program MAGIC. Six methyl groups (2 alanines (A141 and A145) and 4 threonines (T90, T88, T115 and T149)) were unambiguously assigned with both a high percentage of NOE correlation peaks assigned and a high confidence score. Four threonines (T94, T109, T176 and T184) were left either with multiple assignments or an assignment with a low confidence score. However, taking into account the information obtained from the NOE-based assignment, the mutant spectra displaying chemical shift perturbations (Fig. S5) were carefully reanalyzed allowing us to assign the four remaining threonine methyl signals.

Finally, all the 87 methyl groups of HSP90-NTD were assigned using both isoleucine precursor and the newly labelled acetolactate for isoleucine, leucine and valine methyl groups and this mixed NOE/mutants strategy for alanine, methionine and threonine methyl moieties (Fig. 5, Table S2). It can be noted that, there is, indeed, an overlap of two isoleucines (I33/I128) explaining only 86 visible peaks. Even though dynamics in the intermediate regime broaden the backbone resonances of the segment which is covering the ATP binding site [103-115] beyond the detection threshold, the according methyl probes are visible. The assignment of L103, I104, L107, T109, I110, A111 and T115 render this previously invisible region amenable to NMR studies.

Conclusion:

This research reports the synthesis of a new dissymmetric $^{13}C/^{2}H$-labelled acetolactate, a precursor that allows to connect directly, via linear ^{13}C chains, backbone atoms to the pro-*S* methyl groups of leucine and valine residues. This optimized precursor can be combined with isoleucine precursor and $^{2}H/^{13}C$-alanine to enable the transfer of assignment from backbone to methyl groups with only one sample without requiring the correction of $^{1}H/^{2}H$ isotopic chemical shift for the ^{13}C resonances. We expect that this new precursor will ease the assignment of leucine and valine pro-*S* methyl groups of proteins using already assigned backbone resonances as it simplifies the analysis of the NMR experiments. This innovative labelling scheme was applied to the 29 kDa N-terminal domain of human HSP90 protein and to the 87 kDa hetero hexameric prefoldin complex. Using both isoleucine precursor and the newly labelled acetolactate, we managed to simply and efficiently transfer the backbone sequential assignment to all the isoleucine-δ_1, leucine and valine pro-*S* methyl moieties of HSP90-NTD. This allowed us to confirm or correct the residue specific assignment of most isoleucine, leucine and valine methyl groups (2 assignments were corrected for the 49 ILV residues – Table S2) and the stereospecific assignment of prochiral methyl groups (5 stereospecific assignments were inverted for the 29 leucine and valine residues – Table S2). In addition to the full assignment of $I^{\delta1}L^{\delta2}V^{\gamma2}$ methyl groups, we have used a mixed strategy based on mutagenesis and intermethyl NOEs to assign 38 new methyl resonances corresponding to the A^{β} M^{ε} T^{γ} methyl moieties of HSP90-NTD. Hence, we show that, despite extended conformational exchange that impedes the complete backbone assignment, we managed to detect and assign signals for all methyl probes including the ones belonging to the segment covering HSP90 ATP binding site.

Data availability

The FIDs acquired for this study are available in the biological magnetic resonance databank (bmrbig12) and the assignment have been deposited under the BMRB ID: 50786.

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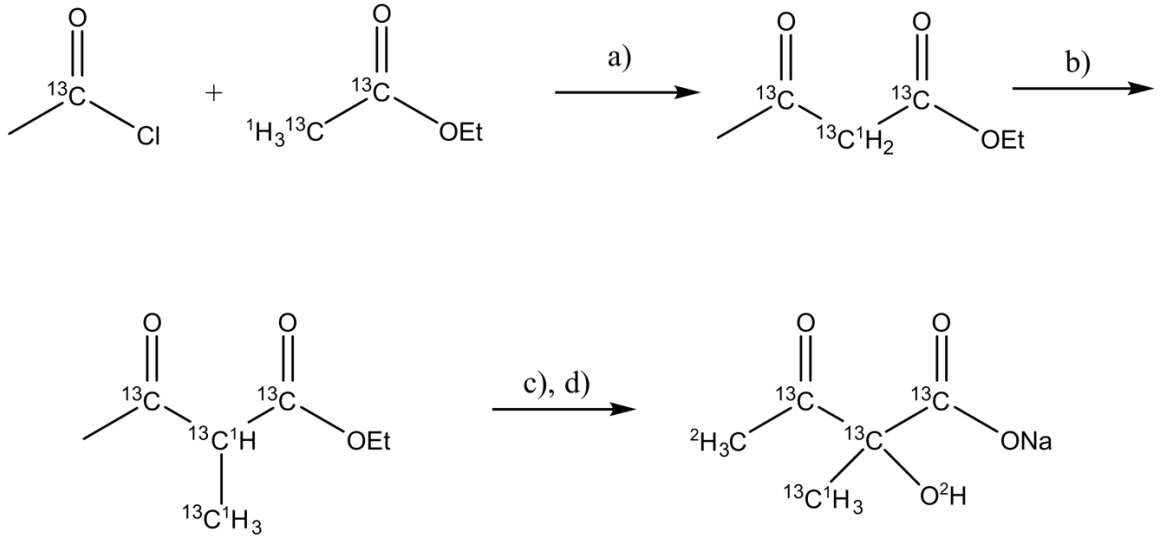

Fig. 1 Synthetic scheme for preparation of specifically labelled sodium 1, 2, 3-[$^{13}C_3$]-2-[$^{13}C^1H_3$]-2-[O^2H]-3-oxo-4, 4, 4-[$^2H_3$]-butanoate. **a)** i-LiHMDS (2.1 equiv.), dry THF, -78°C; ii-1,2-[$^{13}C_2$]-ethyl acetate (1 equiv.), -78°C, 15 min.; iii-1-[^{13}C]-acetyl chloride (1.0 equiv.), -78°C, 30 min.; iv-^{1}HCl 20 %; **b)** K_2CO_3 (1.1 equiv.), $^{13}C^1H_3$-I (1.1 equiv.), 0°C, 18h, EtO^1H; **c)** Cs_2CO_3 (0.2 equiv.), $P(OEt)_3$ (0.2 equiv.), O_2, DMSO, 20h. **d)** i-NaO^2H (2.5M), ^2H_2O; ii-^{2}HCl 35%; Tris buffer pH 7.5; 27 % overall yield.

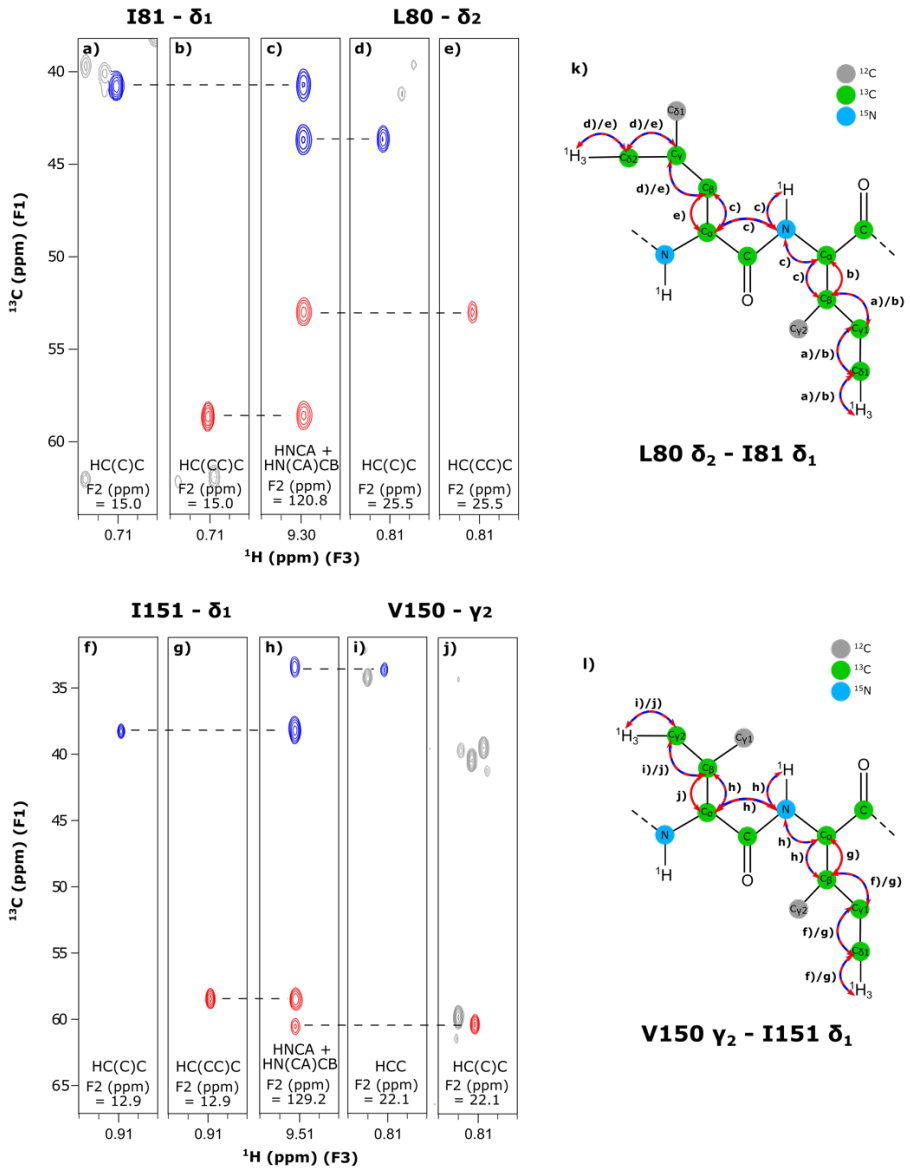

Fig. 2 Assignment transfer from the backbone to Leu$^{pro\text{-}S}$, Val$^{pro\text{-}S}$ and Ile-δ_1 methyl groups of HSP90-NTD. Examples of 2D-extracts from 3D 'out and back' HCC (i), HC(C)C (a, d, f and j) and HC(CC)C (b, e and g) experiments correlating ^{1}H (F_3) and ^{13}C (F_2) methyl resonances with $^{13}C_\beta$ (blue) or $^{13}C_\alpha$ (red) in F_1 dimension. Panels c and h display the corresponding 2D HNCA and HN(CA)CB extracts for Ile-81 (c) and Ile-151 (h) allowing to connect Leu-80-δ_2 (pro-*S*), Ile-81-δ_1, Val-150-γ_2 (pro-*S*), Ile-151-δ_1 methyl groups to previously assigned backbone atoms. 3D spectra were recorded on an NMR spectrometer operating at a proton frequency of 600 MHz using the U-[^{2}H, ^{15}N, ^{13}C], Ile-[2, 3, 4, 4-$^{2}H_4$; 1, 2, 3, 4-$^{13}C_4$; $^{13}C^{1}H_3]^{\delta1}$/[$^{12}C^{2}H_3]^{\gamma2}$], Leu-[2, 3, 3, 4-$^{2}H_4$; 1, 2, 3, 4-$^{13}C_4$; [$^{13}C^{1}H_3]^{pro\text{-}S}$/[$^{12}C^{2}H_3]^{pro\text{-}R}$], Val-[2, 3-$^{2}H_2$; 1, 2, 3-$^{13}C_3$; [$^{13}C^{1}H_3]^{pro\text{-}S}$/[$^{12}C^{2}H_3]^{pro\text{-}R}$] labelled sample or U-[$^{2}H$, ^{15}N, ^{13}C] labelled sample (3D HNCA and HN(CA)CB). (k) and (l) represent magnetization transfer schemes correlating with the strips (a, b, c, d, e) and (f, g, h, i, j), respectively. Except when specified, all hydrogen atoms are ^{2}H.

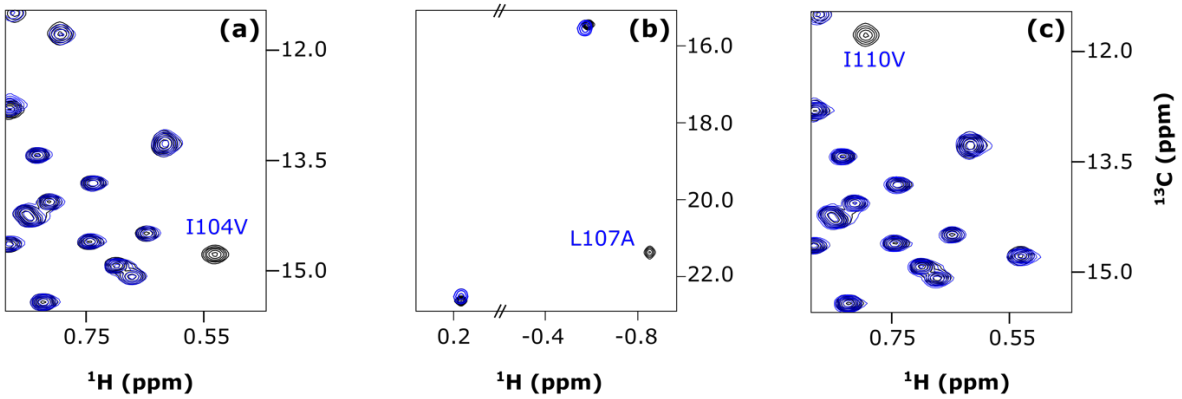

Fig. 3 Assignment of HSP90-NTD methyl groups belonging to the flexible loop covering ATP binding site. The 2D SOFAST methyl TROSY spectra were recorded using either the isoleucine to valine mutant samples using U-[^{2}H, ^{12}C, ^{15}N]-Ile-[^{13}C^{1}H$_3$]$^{\delta1}$ labelling scheme, or the leucine to alanine mutant sample using U-[^{2}H, ^{12}C, ^{15}N]-Leu/Val-[^{13}C^{1}H$_3$]$^{pro\text{-}S}$ labelling scheme. Spectra were recorded at 298 K on a NMR spectrometer operating at a proton frequency of 850 MHz. a) The HSP90-NTD mutant spectra of I104V. b) L107A. c) I110V. Each mutant spectrum extract (dark blue) was superimposed with the wild type protein extract (black).

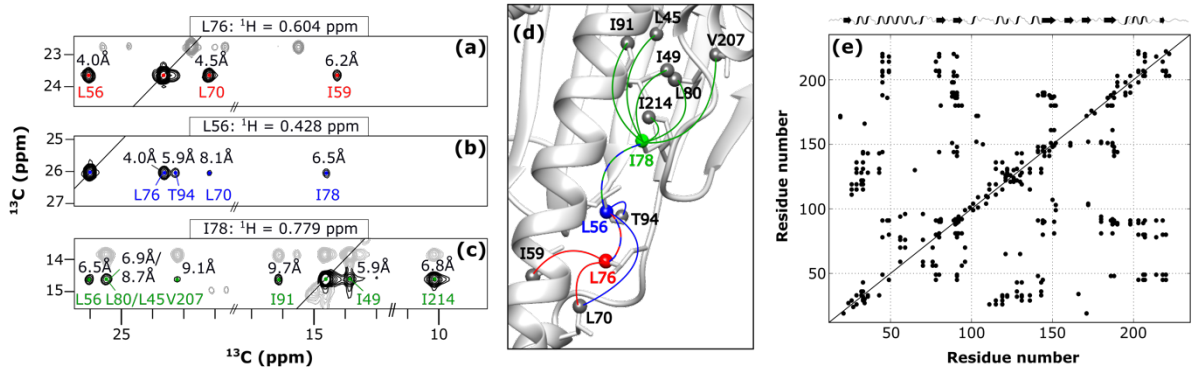

Fig. 4 Detected intermethyl NOEs in human HSP90-NTD. **a-c** Examples of 2D extracts of a 3D HMQC-NOESY-HMQC experiment recorded using U-[^{2}H,^{12}C,^{15}N]-Leu/Val-[^{13}C^{1}H$_3$]$^{pro\text{-}S}$, Ile-[^{13}C^{1}H$_3$]$^{\delta 1}$, Met-[^{13}C^{1}H$_3$]$^{\varepsilon}$, Ala-[^{13}C^{1}H$_3$]$^{\beta}$, Thr-[^{13}C^{1}H$_3$]$^{\gamma}$ HSP90-NTD sample on a NMR spectrometer operating at a proton frequency of 950 MHz. The planes were extracted at the methyl proton frequencies of L76 (a), L56 (b) and I78 (c). The NOEs detected are colored in red, blue and green, respectively. **d** The NOEs detected in 2D extracts presented in panels a-c are displayed on the 3D structure of HSP90-NTD (PDB: 1YES) by lines (red for L76, blue for L56 and green for I78). **e** 2D matrix representing all the HSP90-NTD methyl residue pairs for which NOE cross-peaks have been detected.

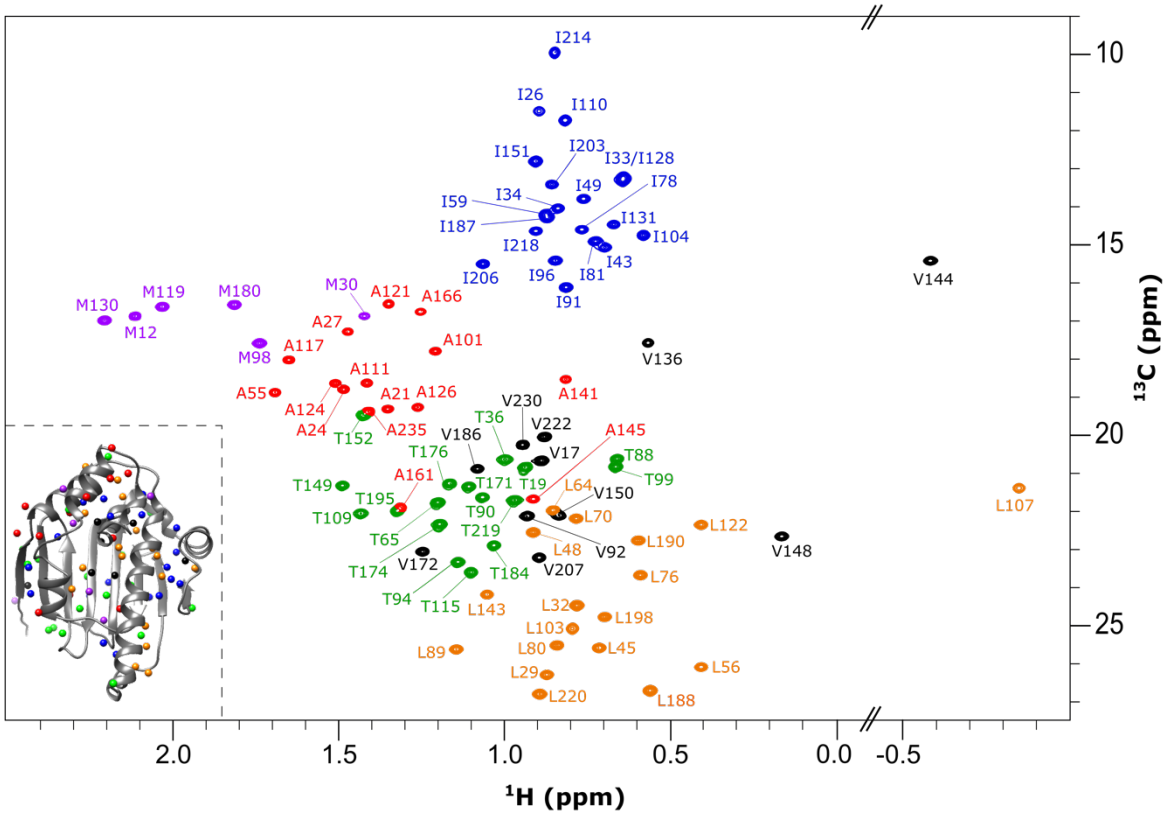

Fig. 5 Assigned 2D ^{1}H-^{13}C SOFAST methyl TROSY spectrum of apo HSP90-NTD. Human HSP90-NTD was perdeuterated and specifically $^{13}C^{1}H_3$-labelled on Leu/Val-[$^{13}C^{1}H_3$]$^{pro\text{-}S}$, Ile-[$^{13}C^{1}H_3$]$^{\delta 1}$, Met-[$^{13}C^{1}H_3$]$^{\varepsilon}$, Ala-[$^{13}C^{1}H_3$]$^{\beta}$, Thr-[$^{13}C^{1}H_3$]$^{\gamma}$ methyl groups. Each signal is annotated with the corresponding residue number. The spectrum was recorded on a NMR spectrometer operating at a proton frequency of 950 MHz. On the bottom left side an insert represents the 3D structure of human HSP90-NTD (PDB: 1YES). The methyl groups are represented by spheres. Alanines, isoleucines, valines, leucines, threonines and methionines are depicted in red, dark blue, black, orange, light green and purple, respectively.

Optimized Precursor to Simplify Assignment Transfer between Backbone Resonances and Stereospecifically labelled Valine and Leucine Methyl Groups: Application to Human Hsp90 N-Terminal Domain

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SUPPLEMENTARY INFORMATION

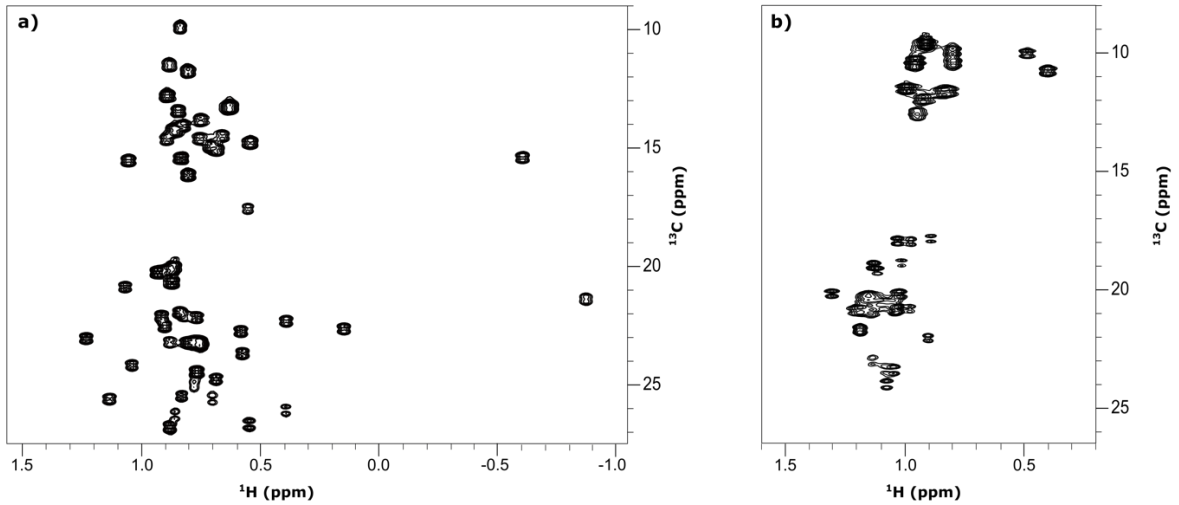

Fig. S1 2D ^{1}H-^{13}C SOFAST methyl TROSY spectrum of U-[^{2}H, ^{15}N, ^{13}C], Ile-[2, 3, 4, 4-$^{2}H_4$; 1, 2, 3, 4-$^{13}C_4$; $[^{13}C^{1}H_3]^{\delta 1}$/$[^{12}C^{2}H_3]^{\gamma 2}$], Leu-[2, 3, 3, 4-$^{2}H_4$; 1, 2, 3, 4-$^{13}C_4$; $[^{13}C^{1}H_3]^{pro\text{-}S}$/$[^{12}C^{2}H_3]^{pro\text{-}R}$], Val-[2, 3-$^{2}H_2$; 1, 2, 3-$^{13}C_3$; $[^{13}C^{1}H_3]^{pro\text{-}S}$/$[^{12}C^{2}H_3]^{pro\text{-}R}$] HSP90-NTD **a)** and prefoldin **b)** samples (labelling scheme A applied to prefoldin β subunit only), recorded before the HCC experiments to check the sample quality. The labelling strategy used Ile-δ_1 precursor: sodium (S)-2-hydroxy-2-(1',1'-[$^{2}H_2$], 1', 2'-[$^{13}C_2$]) ethyl-3-oxo-1,2,3-[$^{13}C_3$]-4,4,4-[$^{2}H_3$]-butanoate) and the suitably labelled acetolactate precursor: 1, 2, 3-[$^{13}C_3$]-2-[$^{13}C^{1}H_3$]-2-[$O^{2}H$]-3-oxo-4, 4, 4-[$^{2}H_3$]-butanoate for the labelling of Leu and valine pro-*S* methyl groups. No additional peaks were detected neither to the pro-*R* methyls groups of leucine and valine nor to the isoleucine-γ_2 site, confirming the absence of isotopic scrambling. The peaks are splitted in the carbon dimension due to the presence of $^{1}J_{CC}$ coupling as expected from the labelling scheme of the produced samples.

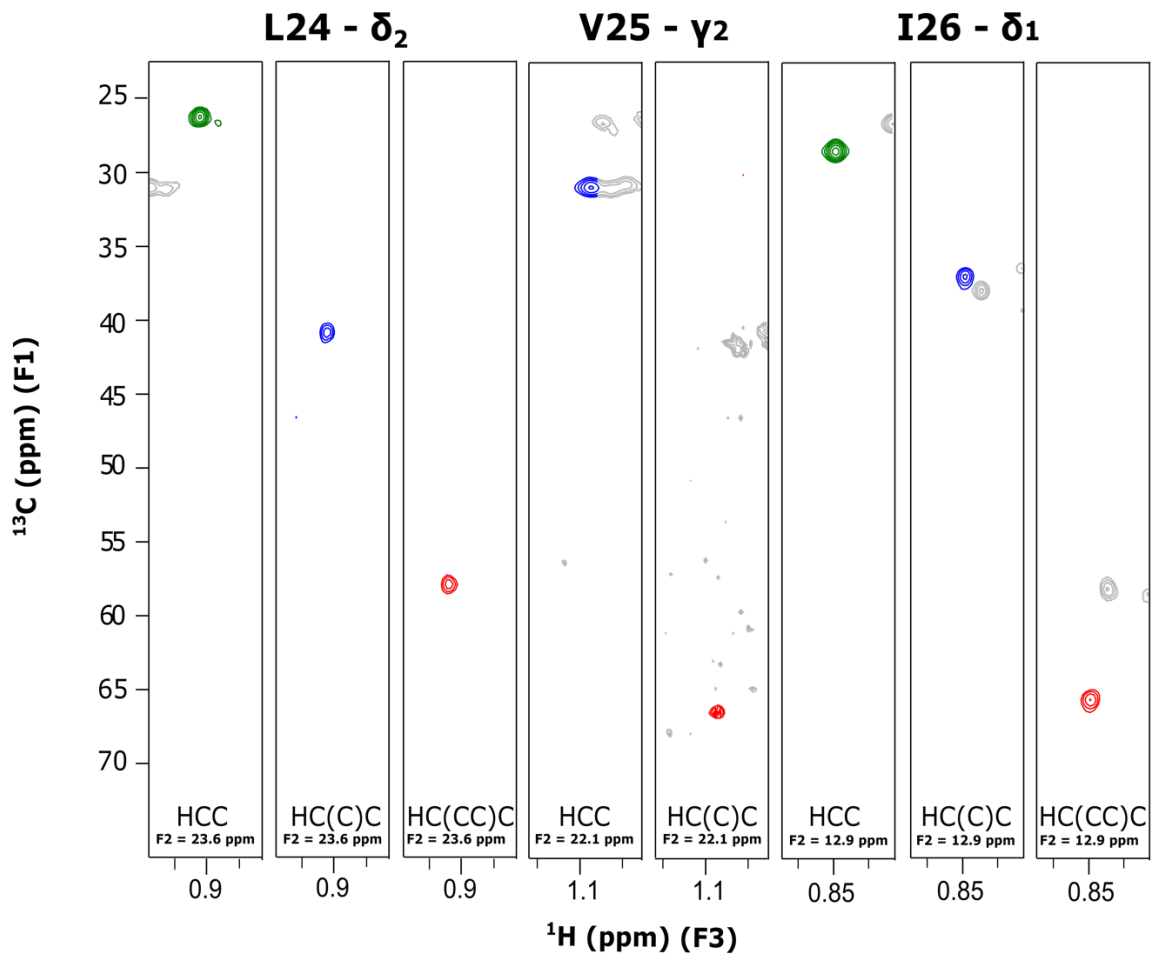

Fig. S2 Application to the 87 kDa prefoldin from *Pyrococcus horikoshii*. Examples of 2D-extracts from 3D 'out and back' HCC , HC(C)C and HC(CC)C experiments correlating ^{1}H (F_3) and ^{13}C (F_2) methyl resonances with $^{13}C_{\gamma}$ (green) $^{13}C_{\beta}$ (blue) or $^{13}C_{\alpha}$ (red) in F_1 dimension of Leu-24, Val-25 and Ile-26 of *Pyrococcus horikoshii* prefoldin subunit β. Spectra have been recorded at 310 K using 0.2 mM U-[^{2}H, ^{15}N, ^{13}C], Ile-[2, 3, 4, 4-$^{2}H_4$; 1, 2, 3, 4-$^{13}C_4$; $^{13}C^{1}H_3]^{\delta1}$/[$^{12}C^{2}H_3]^{\gamma2}$], Leu-[2, 3, 3, 4-$^{2}H_4$; 1, 2, 3, 4-$^{13}C_4$; [$^{13}C^{1}H_3]^{pro\text{-}S}$/[$^{12}C^{2}H_3]^{pro\text{-}R}$], Val-[2, 3-$^{2}H_2$; 1, 2, 3-$^{13}C_3$; [$^{13}C^{1}H_3]^{pro\text{-}S}$/[$^{12}C^{2}H_3]^{pro\text{-}R}$] sample of prefoldin on an NMR spectrometer operating at a proton frequency of 950 MHz. The acquisition parameters were similar to that used for HSP90-NTD, described in Materials and Methods section. Prefoldin was expressed and purified according to the protocol described in Törner et al. (2020) and labelled on the β subunit only using labelling scheme A, described in Materials and Methods section of the present manuscript.

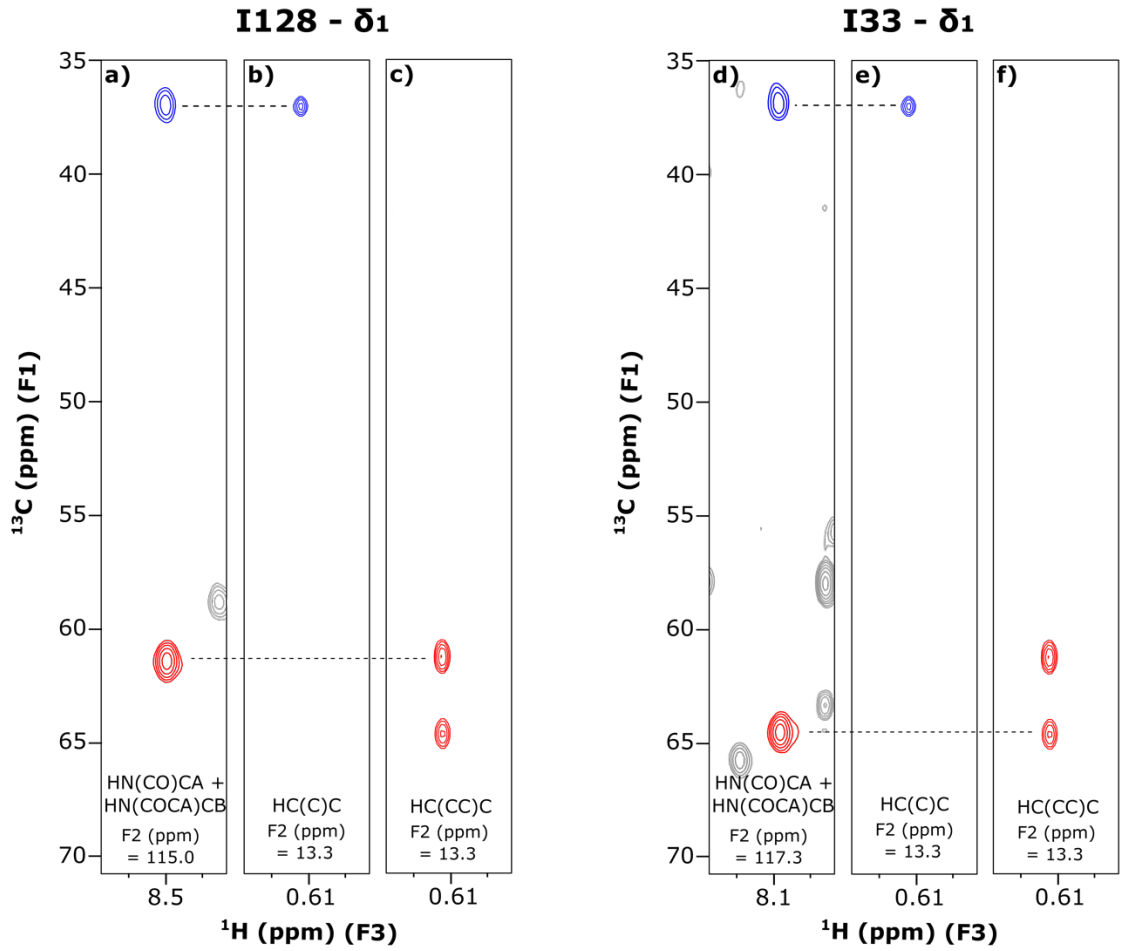

Fig. S3 Assignment transfer from the backbone to the Ile-δ_1 methyl groups of Ile-33 and Ile-128 of HSP90-NTD. Examples of 2D-extracts from 3D 'out and back' HC(C)C (b and e) and HC(CC)C (c and f) experiments correlating ^{1}H (F_3) and ^{13}C (F_2) methyl resonances with $^{13}C_\beta$ (blue) or $^{13}C_\alpha$ (red) in F_1 dimension. Panels a and d display the corresponding 2D HN(CO)CA and HN(COCA)CB extracts for Ile-128 (a) and Ile-33 (d) allowing to connect Ile-33-δ_1 and Ile-128-δ_1 methyl groups to previously assigned backbone atoms. 3D spectra were recorded on an NMR spectrometer operating at a proton frequency of 600 MHz using the U-[^{2}H, ^{15}N, ^{13}C], Ile-[2, 3, 4, 4-$^{2}H_4$; 1, 2, 3, 4-$^{13}C_4$; $^{13}C^{1}H_3]^{\delta1}$/[$^{12}C^{2}H_3]^{\gamma2}$], Leu-[2, 3, 3, 4-$^{2}H_4$; 1, 2, 3, 4-$^{13}C_4$; [$^{13}C^{1}H_3]^{pro\text{-}S}$/[$^{12}C^{2}H_3]^{pro\text{-}R}$], Val-[2, 3-$^{2}H_2$; 1, 2, 3-$^{13}C_3$; [$^{13}C^{1}H_3]^{pro\text{-}S}$/[$^{12}C^{2}H_3]^{pro\text{-}R}$] labelled sample or U-[$^{2}H$, ^{15}N, ^{13}C] labelled sample (3D HN(CO)CA and HN(COCA)CB).

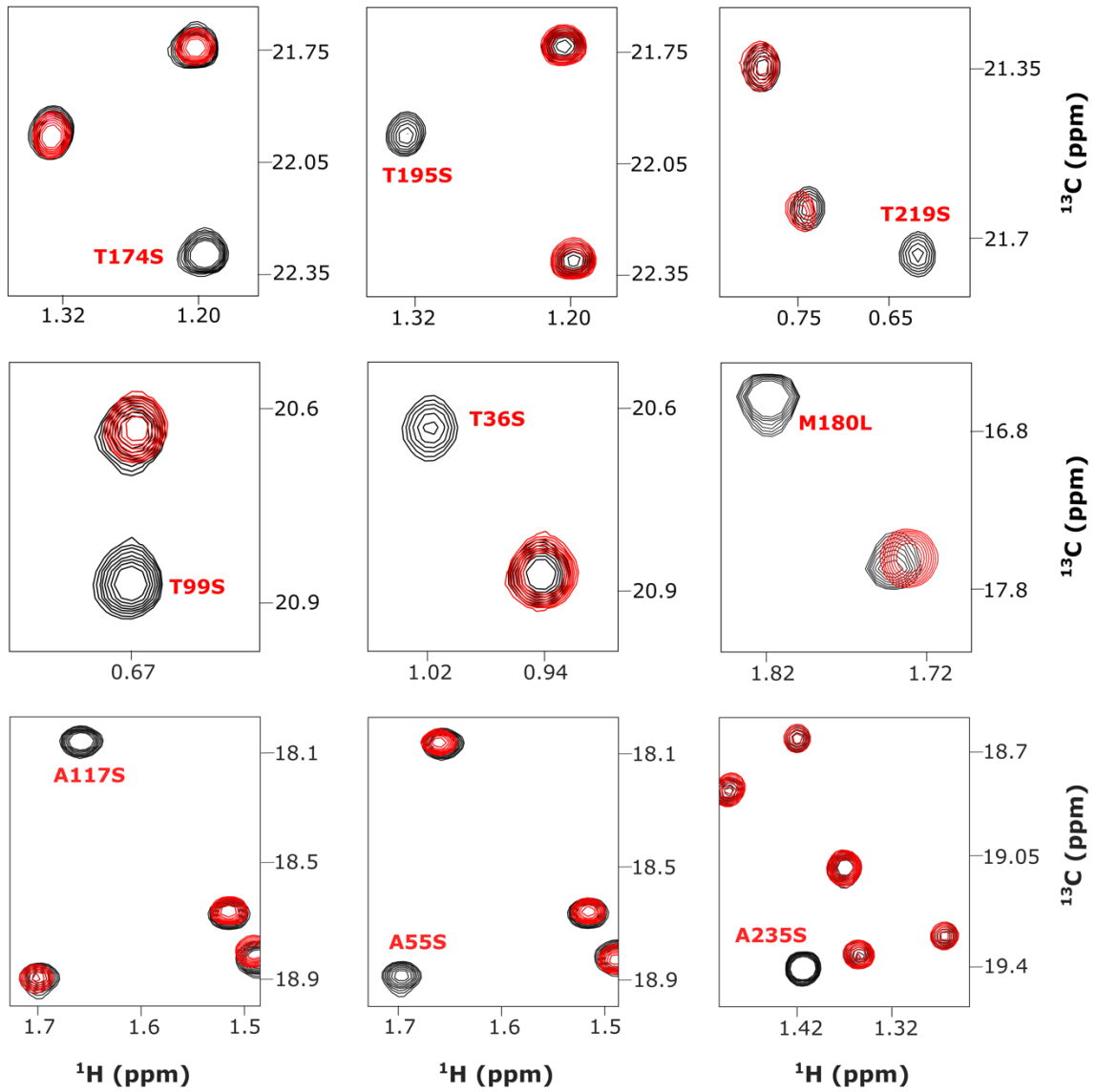

Fig. S4 Assignment of methyl groups using mutagenesis. Examples of 2D ^{1}H-^{13}C SOFAST methyl TROSY spectra of HSP90-NTD mutants, for which no or minor chemical shift perturbations are observed. Spectra were acquired on an NMR spectrometer operating at a proton frequency of 850 MHz. Each mutant spectrum extract (colored) is superimposed with the corresponding spectra acquired using wild type HSP90-NTD methyl labelled spectra (black). The signal corresponding to each mutant is annotated in color. List of the mutants produced for this study: M12L, M30L M119L, M130L, M180L, A21S, A24S, A27S, A55S, A101S, A111S, A117S, A121S, A124S, A126S, A161S, A166S, A235S, T19S, T36S, T65S, T99S, T109S, T115S, T152S, T171S, T174S, T184S, T195S, T219S, I104V, L107A, I110V.

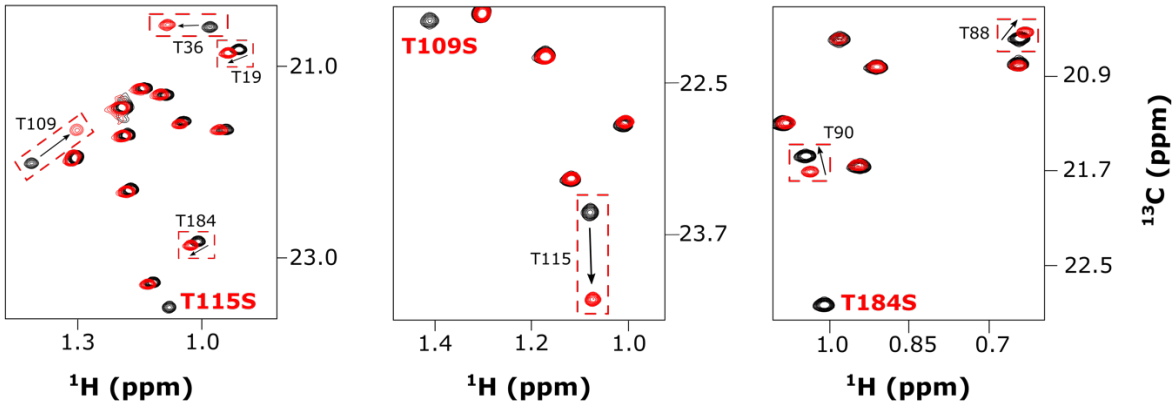

Fig. S5 Assignment of methyl groups using mutagenesis. Examples of 2D ^{1}H-^{13}C SOFAST methyl TROSY spectra of HSP90-NTD mutants, for which chemical shift perturbations. Spectra were acquired on an NMR spectrometer operating at a proton frequency of 850 MHz. Each mutant spectrum extract (colored) is superimposed with the corresponding spectra acquired using wild type HSP90-NTD methyl labelled spectra (black). The signal corresponding to each mutant is annotated in color. Black arrows represent secondary chemical shifts.

Table S1: Output file from MAGIC software for the assignment of HSP90-NTD methyl groups.

The 1st column displays the best assignment. The 2nd and 3rd columns give the ^{13}C and ^{1}H chemical shifts, respectively. The 4th column represents the sum of all peak-peak connection confident scores for each peak. The 5th column shows the NOE assignment completeness of the strip related to each peak and the 6th column displays the list of all possible assignment with their scores (Monneau et al. 2017).

The automated methyl assignment was performed using the reference structure of HSP90-NTD (PDB: 1YES). MAGIC was run with a score threshold factor of 1, distance thresholds of 7–10 Å and using the 344 inter methyl NOE cross peaks (S/N ≥ 5) detected (Fig. 4e). In addition, MAGIC was given the methyl type for each methyl group, previously identified using specific methyl labelling. In blue are the methyl groups assigned automatically by MAGIC that have 1) a single assignment, 2) a high NOE assignment completeness of the strip related to each peak (> 50 %) and 3) a high total confidence score value (≥ 7).

Predicted Assignment	^{13}C ppm	^{1}H ppm	Score	% NOE	Possible Assignment
V144CG*-HG*	15.487	-0.566	31.48	0.86	{'V144CG': 215.504}
L56CD*-HD*	26.036	0.428	9.0	1.0	{'L56CD': 215.504}
L89CD*-HD*	25.59	1.159	26.22	1.0	{'L89CD': 215.504}
L45CD*-HD*	25.544	0.731	36.08	1.0	{'L80CD': 207.896 'L45CD': 215.504}
L80CD*-HD*	25.474	0.86	33.17	1.0	{'L80CD': 215.504 'L45CD': 207.896}
L198CD*-HD*	24.747	0.717	15.0	1.0	{'L198CD': 215.504}
L32CD*-HD*	24.451	0.802	13.67	0.8	{'L32CD': 215.504}
L143CD*-HD*	24.158	1.061	13.0	1.0	{'L143CD': 215.504 'L122CD': 206.867}
L76CD*-HD*	23.647	0.605	9.0	1.0	{'L70CD': 207.413 'L76CD': 215.504}
T94CG2-HG2	23.292	1.157	7.0	1.0	{'T184CG2': 206.767 'T152CG2': 207.402 'T94CG2': 215.504}
T115CG2-HG2	23.566	1.118	24.6	1.0	{'T115CG2': 215.504}
M130CE-HE	17.026	2.213	5.0	0.25	{'M130CE': 215.504}
V207CG*-HG*	23.223	0.91	27.69	1.0	{'V207CG': 215.504}
V172CG*-HG*	23.024	1.257	3.0	0.5	{'V172CG': 215.504}
L190CD*-HD*	22.76	0.615	33.53	1.0	{'L190CD': 215.504}
V148CG*-HG*	22.646	0.178	24.0	0.83	{'V148CG': 215.504}
L48CD*-HD*	22.527	0.93	19.5	0.67	{'L48CD': 215.504}
T19CG2-HG2	22.331	1.204	2.0	0.0	{'T19CG2': 215.504 'T176CG2': 215.504 'T174CG2': 215.504 'T171CG2': 215.504}
V92CG*-HG*	22.125	0.946	27.35	1.0	{'V92CG': 215.504}
L70CD*-HD*	22.186	0.801	10.39	1.0	{'L70CD': 215.504 'L76CD': 207.413}
I214CD1-HD1	10.135	0.86	13.38	1.0	{'I78CD1': 207.021 'I214CD1': 215.504}
I26CD1-HD1	11.564	0.908	14.0	1.0	{'I26CD1': 215.504}
I110CD1-HD1	11.885	0.831	2.0	1.0	{'I110CD1': 215.504}
I151CD1-HD1	12.894	0.916	15.0	0.57	{'I151CD1': 215.504}
I33CD1-HD1	13.355	0.661	36.6	1.0	{'I33CD1': 215.504}
I203CD1-HD1	13.467	0.874	33.35	1.0	{'I203CD1': 215.504}
I49CD1-HD1	13.848	0.775	34.45	0.86	{'I49CD1': 215.504}

I34CD1-HD1	14.091	0.859	2.0	1.0	{'I34CD1':	215.504}			
I187CD1-HD1	14.32	0.88	12.37	1.0	{'I187CD1':	215.504}			
I59CD1-HD1	14.25	0.89	8.37	0.75	{'I96CD1':	207.538	'I59CD1':	215.504}	
I131CD1-HD1	14.458	0.688	23.0	0.86	{'I131CD1':	215.504}			
I78CD1-HD1	14.622	0.779	24.0	1.0	{'I218CD1':	207.374	'I78CD1':	215.504	
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M119CE-HE	16.667	2.047	26.67	0.71	{'M119CE':	215.504}			
I218CD1-HD1	14.66	0.916	22.0	0.8	{'I218CD1':	215.504	'I78CD1':	207.374	
					'I214CD1':	206.692}			
I104CD1-HD1	14.808	0.565	4.0	1.0	{'I104CD1':	215.504}			
I81CD1-HD1	14.949	0.733	42.89	0.45	{'I81CD1':	215.504}			
I43CD1-HD1	15.1	0.714	10.0	1.0	{'I43CD1':	215.504	'I128CD1':	208.077}	
I96CD1-HD1	15.467	0.865	5.92	0.67	{'I96CD1':	215.504	'I59CD1':	207.538}	
I206CD1-HD1	15.535	1.076	28.33	0.83	{'I206CD1':	215.504}			
I91CD1-HD1	16.128	0.832	77.51	0.81	{'I91CD1':	215.504}			
A124CB-HB	16.617	1.357	6.0	0.67	{'A121CB':	215.504	'A126CB':	207.223	
					'A124CB':	215.504}			
A166CB-HB	16.771	1.262	2.0	1.0	{'A166CB':	215.504}			
A27CB-HB	17.326	1.486	2.0	1.0	{'A27CB':	215.504	'A24CB':	215.504}	
V136CG*-HG*	17.675	0.576	19.67	1.0	{'V136CG':	215.504}			
A101CB-HB	17.824	1.225	3.0	1.0	{'A101CB':	215.504}			
A141CB-HB	18.538	0.827	15.0	1.0	{'A145CB':	207.062	'A141CB':	215.504}	
A121CB-HB	18.655	1.528	4.33	1.0	{'A121CB':	215.504	'A117CB':	207.058	
					'A126CB':	207.223	'A124CB':	215.504}	
A111CB-HB	18.69	1.429	5.0	1.0	{'A111CB':	215.504	'A27CB':	207.455	
					'A24CB':	207.059}			
A24CB-HB	18.812	1.5	2.0	1.0	{'A27CB':	215.504	'A24CB':	215.504}	
A126CB-HB	19.272	1.272	9.83	1.0	{'A121CB':	207.223	'A126CB':	215.504}	
M180CE-HE	16.604	1.829	29.55	0.64	{'M180CE':	215.504}			
T152CG2-HG2	19.402	1.427	3.23	0.4	{'T152CG2':	215.504}			
V222CG*-HG*	20.053	0.894	5.0	1.0	{'V222CG':	215.504}			
T36CG2-HG2	20.646	1.008	8.0	1.0	{'T36CG2':	215.504}			
T88CG2-HG2	20.629	0.675	8.0	0.75	{'T88CG2':	215.504	'T195CG2':	207.358}	
T99CG2-HG2	20.83	0.676	2.0	0.33	{'T99CG2':	215.504}			
L220CD*-HD*	26.75	0.906	12.31	1.0	{'L220CD':	215.504}			
V186CG*-HG*	20.862	1.1	19.26	0.57	{'V150CG':	207.024	'V186CG':	215.504}	
T176CG2-HG2	21.263	1.172	2.0	0.0	{'T171CG2':	215.504	'T176CG2':	215.504	
					'T174CG2':	215.504	'T19CG2':	215.504}	
T149CG2-HG2	21.331	1.498	9.0	1.0	{'T149CG2':	215.504}			
A145CB-HB	21.67	0.932	16.27	1.0	{'A145CB':	215.504	'A141CB':	207.062}	
T90CG2-HG2	21.638	1.078	29.0	0.71	{'T90CG2':	215.504}			
T195CG2-HG2	21.988	1.34	5.0	1.0	{'T195CG2':	215.504	'T88CG2':	207.358}	
L188CD*-HD*	26.645	0.575	38.1	0.78	{'L188CD':	215.504}			
T109CG2-HG2	22.02	1.444	3.0	1.0	{'T109CG2':	215.504}			
L122CD*-HD*	22.419	0.431	8.1	0.75	{'L143CD':	206.867	'L122CD':	215.504}	
L103CD*-HD*	24.974	0.797	2.73	1.0	{'L103CD':	215.504	'L107CD':	207.733	
					'L64CD':	207.687}			
A161CB-HB	21.9	1.327	7.0	0.67	{'A161CB':	215.504}			

L29CD*-HD*	26.252	0.89	27.0	1.0	{'L29CD': 215.504}
A55CB-HB	19.402	1.427	3.23	0.0	{'A24CB': 208.147 'A166CB': 208.147 'A21CB': 215.504 'A111CB': 207.455 'A27CB': 208.147 'A117CB': 215.504 'A235CB': 215.504 'A55CB': 215.504 'A124CB': 207.058}
T65CG2-HG2	20.867	0.944	2.0	0.0	{'T171CG2': 215.504 'T176CG2': 215.504 'T174CG2': 215.504 'T65CG2': 215.504 'T19CG2': 215.504 'T219CG2': 215.504}
T184CG2-HG2	21.357	1.117	2.0	1.0	{'T171CG2': 207.047 'T152CG2': 207.267 'T94CG2': 206.547 'T176CG2': 207.047 'T174CG2': 207.047 'T65CG2': 207.047 'T19CG2': 207.047 'T219CG2': 207.047 'T184CG2': 215.504}
T219CG2-HG2	21.756	1.216	3.0	0.0	{'T171CG2': 215.504 'T152CG2': 208.147 'T94CG2': 207.402 'T176CG2': 215.504 'T174CG2': 215.504 'T65CG2': 215.504 'T19CG2': 215.504 'T219CG2': 215.504 'T184CG2': 207.267}

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Not Assigned	**^{13}C ppm**	**^{1}H ppm**	
3	16.949	2.087	NotAss ['M30CE' 'M98CE' 'M12CE']
5	17.58	1.732	NotAss ['M30CE' 'M98CE' 'M12CE']
49	16.898	1.43	NotAss ['M30CE' 'M98CE' 'M12CE']
53	18.034	1.659	NotAss ['A21CB' 'A117CB' 'A235CB']
58	18.938	1.694	NotAss ['A21CB' 'A117CB' 'A235CB']
60	19.325	1.362	NotAss ['A21CB' 'A117CB' 'A235CB']
64	20.266	0.959	NotAss ['V17CG' 'V150CG' 'V230CG']
67	20.699	0.9	NotAss ['V17CG' 'V150CG' 'V230CG']
73	21.392	-0.838	NotAss ['L64CD' 'L107CD']
75	21.709	0.974	NotAss ['T171CG2' 'T174CG2']
80	21.965	0.865	NotAss ['L64CD' 'L107CD']
82	22.208	0.844	NotAss ['V17CG' 'V150CG' 'V230CG']
86	22.86	1.022	NotAss ['T171CG2' 'T174CG2']

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Table S2: Assignment of HSP90-NTD methyl groups

Met	H(ε)	C(ε)
M12	2.117	16.889
M30	1.426	16.886
M98	1.741	17.594
M119	2.035	16.644
M130	2.210	17.000
M180	1.818	16.593

Thr	H(γ2)	C(γ2)
T19	0.942	20.832
T36	1.002	20.622
T65	1.205	21.746
T88	0.664	20.605
T90	1.07	21.611
T94	1.144	23.293
T99	0.667	20.819
T109	1.437	22.033
T115	1.104	23.567
T149	1.492	21.304
T152	1.430	19.470
T171	1.111	21.333
T174	1.199	22.314
T176	1.169	21.250
T184	1.036	22.863
T195	1.326	21.960
T219	0.973	21.686

Ala	H(β)	C(β)
A21	1.356	19.310
A24	1.489	18.799
A27	1.476	17.297
A55	1.696	18.870
A101	1.211	17.806
A111	1.419	18.635
A117	1.654	18.031
A121	1.353	16.570
A124	1.514	18.640
A126	1.265	19.266
A141	0.819	18.528
A145	0.917	21.654
A161	1.318	21.910
A166	1.257	16.777
A235	1.415	19.384

Ile	H(δ_1)	C(δ_1)
I26[b]	0.899	11.547
I33/I128[a]	0.647	13.317
I34[a]	0.842	14.078
I43[a]	0.702	15.097
I49[a]	0.765	13.830
I59[a]	0.876	14.287
I78[a]	0.771	14.631
I81[a]	0.729	14.935
I91[a]	0.818	16.139
I96[a]	0.850	15.440
I104[a]	0.585	14.778
I110[b]	0.821	11.783
I131[a]	0.675	14.498
I151[a]	0.910	12.853
I187[a]	0.876	14.287
I203[a]	0.861	13.455
I206[a]	1.069	15.528
I214[a]	0.853	10.015
I218[a]	0.909	14.670

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Leu	H(δ_2)	C(δ_2)
L29[c]	0.876	26.240
L32[c]	0.786	24.429
L45[d]	0.719	25.537
L48[c]	0.918	22.526
L56[c]	0.411	26.037
L64[c]	0.853	21.951
L70[c]	0.787	22.168
L76[c]	0.594	23.642
L80[c]	0.846	25.472
L89[c]	1.149	25.579
L103[c]	0.798	25.037
L107[d]	-0.851	21.374
L122[c]	0.411	22.329
L143[d]	1.056	24.148
L188[d]	0.565	26.652
L190[c]	0.601	22.739
L198[c]	0.702	24.73
L220[c]	0.898	26.742

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Val	H(γ_2)	C(γ_2)
V17[d]	0.892	20.658
V92[c]	0.936	22.102
V136[c]	0.570	17.586
V144[c]	-0.582	15.446

V148[c]	0.168	22.630
V150[c]	0.839	22.084
V172[c]	1.251	23.028
V186[c]	1.085	20.870
V207[c]	0.899	23.183
V222[c]	0.884	20.037
V230	0.949	20.244

[a] Residues assigned by (Park et al. 2011) and confirmed by this study.
[b] Assignment was inverted for these two residues in our study compared to (Park et al. 2011).
[c] Residues stereospecifically assigned by (Lescanne et al. 2018) and confirmed by this study.
[d] Stereospecific assignment was inverted compared to (Lescanne et al. 2018).