Asymmetric Synthesis of Methyl Specifically Labelled L -Threonine and Application to the NMR Studies of High Molecular Weight Proteins

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Asymmetric Synthesis of Methyl Specifically Labelled *L*-Threonine and Application to the NMR Studies of High Molecular Weight Proteins

Isabel Ayala[+],[a] Lucile Chiari[+],[b] Rime Kerfah,[c] Jerome Boisbouvier,*[a] Pierre Gans,[a] and Olivier Hamelin*[b]

Methyl groups are valuable probes for solution NMR, allowing the investigation of large protein complexes. Among the six methyl containing residues, threonine has one of the less hydrophobic side chain, and can reside both within the interior of a protein or on the protein surface. This article presents an efficient mixed chemical/enzymatic synthesis scheme, enabling the preparation of threonine with natural configuration on the two stereogenic centers together with an optimal introduction of $^1H/^2H$ and $^{12}C/^{13}C$ atoms at specific sites. Such specifically labelled amino acid can be efficiently incorporated in overexpressed proteins without scrambling or in combination with any other types of $^{13}CH_3$ probes. Additionally we report application to the 82 kDa Malate Synthase G. Our findings demonstrate that structural meaningful long range nOes can be detected between threonine methyl probes and methionine and isoleucine methyl groups distant by 12 Å.

1. Introduction

The use of solution NMR spectroscopy to analyze the dynamics, interactions and function of large proteins (>100 kDa) is becoming increasingly feasible.[1] Key to these advances has been the development of protocols for the expression of selectively methyl-protonated perdeuterated proteins.[2] Methyl groups have been proven to be ideal molecular probes for solution NMR spectroscopy studies of large proteins. Several protocols for selective protonation of hydrophobic methyl containing amino-acids Ile-δ1,[3] Ile-γ2,[4] Leu-δ1/2 and Val-γ1/2,[5] Ala-β,[6] and Met-ε[7] methyl groups have been proposed. Protocols for the stereospecific protonation of Leu-δ and/or Val-γ methyl groups were also reported.[8] In each of these labelling schemes an isotope-labelled metabolic precursor or amino acid (Ala or Met) is added to a perdeuterated expression culture, which is then incorporated into the target protein by the bacteria without detectable scrambling. NMR signals of protonated methyl groups can be exploited as sensitive site-specific probes for the characterization of intermolecular interactions,[1a] dynamics on a wide range of time-scales[2b] or the detection of important structural restraints between hydrophobic residues located in protein core.[2c]

Threonine is an interesting methyl containing amino-acids, as this side chain forms both hydrogen bonds and non-polar interactions. Therefore, this residue can be found either in the core of the protein but also at the protein surface.[9] For threonine, complete $^{13}CH_3$ labelling in a deuterated context appears to be more difficult using precursors in the biosynthetic pathway. *In-vivo*, threonine is synthesized from the Krebs cycle via Aspartate by a cascade of enzymatic reactions. As aspartate is a compound involved in the biosynthesis pathways of several amino acids, it is not possible to selectively label threonine from labelled carbon sources without addition of several deuterated amino acids in order to dilute the isotopic leaks.[9] In any case, complete incorporation of $^{13}CH_3$ labelled threonine cannot be attained from these conventional labelled carbon sources. The best $^{13}CH_3$ labelling efficiency obtained from a mixture of 2-^{13}C-glycerol and ^{13}C bicarbonate does not exceed 60% in ^{13}C.[9] The addition of labelled homoserine, the precursor of threonine in its biosynthetic pathway is also not a solution, as homoserine conversion by the threonine synthase in deuterated medium is not selective and results in the production of Thr isotopomers in Cγ position.[10] Consequently, full labelling required the direct use of $^{13}CH_3$-labelled *L*-Thr deuterated in α/β positions. Using a biosynthetic strategy of five enzymatic steps, this compound was produced with 60% yield from ^{13}C-labelled formaldehyde and pyruvate.[11] Despite the elegance of this protocol and the low cost of isotopic-enriched starting materials, this protocol is time-consuming, requiring overproduction and purification of five different enzymes. Although specifically $^{13}CH_3$-labelled threonine is commercially available, here, we propose an alternative chemical route to obtain suitably $^{13}CH_3$-labeled threonine for NMR studies of large proteins. An efficient scheme for the chemical synthesis of a $^{13}CH_3$-isotopically-labelled *L*-threonine in six steps and its selective biosynthetic incorporation into perdeuterated

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proteins is proposed. In addition, we also describe protocols allowing the simultaneous labelling of Thr-$\gamma2$ methyl groups with any combination of other methyl containing amino acids. We demonstrate that the simultaneous labelling of Isoleucine-δ_1, Methionine-ε and Threonine-γ_2 methyl groups allows to reduce signal overlaps in 2D methyl TROSY of large proteins. Furthermore, this combinatorial labelling allows to extract precious intermethyl nOe-distance restraints between methyl probes distant by 12 Å in the 82 kDa Malate Synthase G.

## 2. Results and Discussion

2.1. Threonine synthesis

The chemical synthesis of specifically labelled *L*-threonine is particularly challenging due to the presence of two stereogenic centers, as well as to the main difficulty in effectively introducing the desired isotopes (^{13}C, ^{2}H) at specific sites with high selectivity. *In-vivo*, *L*-phosphohomoserine is converted in the desired amino acid by threonine synthetase (TS, EC# 4.2.3.1). As the chemical synthesis of phosphohomoserine has been previously described, it could have been envisaged to use it as a precursor and to convert it enzymatically to threonine.[11,12] However, the protocols require several consecutive steps, which are difficult to set up with an acceptable performance required for the preparation of isotopically labelled precursors. Subsequently it was decided to directly synthesize the labelled *L*-Thr amino acid.[13] A highly stereoselective reaction was set up relying on the condensation of acetyl chloride on chiral glycine derivative, as previously proposed for the asymmetric synthesis of (2S,3S)-4-fluorothreonine.[14] This protocol was modified in order to selectively introduce ^{1}H, ^{2}H and ^{13}C at specific positions (Figure 1).

Due to enolizable positions on several synthetic intermediates (i.e. intermediates 3, 4 and 5), the reported synthesis proved to be very challenging. While incorporation of a ^{13}C at the threonine-γ position was not a problem using commercially available ^{13}C acetyl chloride, the introduction of ^{2}H and ^{1}H nuclei at the Cα and Cγ positions, respectively, was the major difficulty due to their enolizable properties resulting in a potential ^{1}H/^{2}H exchange with solvent. It was initially thought that the ^{2}H located at the Cα could be stereoselectively introduced during the final step of the acidic cleavage of the chiral auxiliary. However, this approach proved to be unsuccessful since no ^{1}H/^{2}H exchange could be observed using ^{2}HCl. Consequently, deuterium had to be introduce at an earlier step. The final gram scale synthesis involves 6 steps with a reasonable overall yield of 37% starting from the commercially available chiral auxiliary (2R)-(−)-2-(*tert*-butyl)-3-methyl-4-imidazolidinone trifluoroacetic acid (**1**) (Figure 1).

Compound **3** is efficiently obtained in two steps from **1** after standard benzoylation[13c] and subsequent Cα deuteration in basic conditions in CH$_3$O^{2}H. Surprisingly, acylation using the reported conditions (−100 °C, 5 min.) enables the desired product with low yield. However, it was found that performing the reaction at a higher temperature (−78 °C) and for a longer time (45 min.) resulted in a yield improvement without affecting the stereoselectivity of the reaction since only one isomer (**4**) could be detected. Introduction of ^{2}H at the Cβ position was then stereoselectively achieved using NaB^{2}H$_4$ as reducing agent in CH$_3$OH yielding a mixture of two isomers (**5** and **5′**, 1:2 ratio) as a consequence of an incomplete migration of the benzoyl group. Unfortunately, such condition lead to the formation of a non-negligible amount of the Cα protonated product due to the ^{2}H/^{1}H exchange in that position. Using CH$_3$O^{2}H as solvent to avoid this protonation was thus tested, but unexpectedly, the problem was shifted to the enolizable C$_\gamma$ position of **4** resulting in the formation of an inseparable mixture of isotopomers (^{13}CH$_3$, ^{13}CH$_2$^{2}H, ^{13}CH^{2}H$_2$ and ^{13}C^{2}H$_3$). Despite numerous efforts (i.e. variation of temperature, sequential addition of the reducing agent…etc.) conditions yielding satisfactory and reproducible results were not found. Therefore, we decided to engage the mixture of **5** and **5′** issued from reduction in CH$_3$OH, in the last step of threonine synthesis. After 72 h in concentrated HCl solution, labelled *L*-threonine was finally obtained in the presence of 7% of allo-threonine. Pure threonine was obtained after column chromatography and subsequent recrystallization in ethanol in reasonable overall yield (37%). Using this protocol, the cost of isotopically labeled reagents (mainly 2-[^{13}C]-acetyl chloride) required to prepare 1 gram of methyl specifically labeled Threonine is lower than USD $600.

As stated above, it was not possible to chemically achieve 100% of deuteration on the Cα. In order to overcome this problem, we used the previously described *E. coli* branched-chain amino acid transferase (BCAT) that catalyzes ^{1}H/^{2}H exchange specifically on the threonine Cα position.[11] The selective labelling and the absolute configuration were confirmed by NMR and Circular Dichroism analysis respectively of the product by comparison with commercially pure *L*-threonine (Figure S1). It is important to note that such a strategy can be extended to the synthesis of various threonine labelling schemes with specific ^{13}C and ^{2}H labelling (i.e ^{13}CH$_2$D or ^{13}CHD$_2$ isotopomers) using appropriate labelled acetyl chloride (or acid
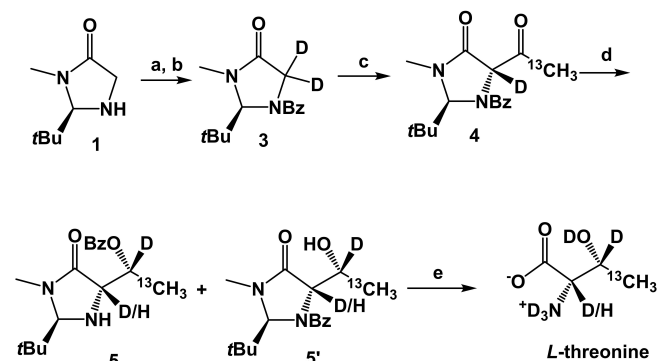
Figure 1. Scheme of the chemical synthesis of the *L*-2-[^{2}H/^{1}H]-3-[^{2}H] 4-[^{13}C]-threonine. a- BzCl, Et$_3$N, CH$_2$Cl$_2$, 15 h; b- K$_2$CO$_3$, CH$_3$O^{2}H, 60 °C, 3 h (89% over two steps); c- i) LDA, THF, −78 °C, 45 min, ii) ^{13}CH$_3$COCl, THF, −78 °C, 1 h (72%); d- NaB^{2}H$_4$, CH$_3$OH, 0 °C ; e- 10 M HCl, reflux, 72 h, Dowex purification then recrystallization (58% over two steps; 37% overall yield).

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derivatives including an additional step) as starting reagent. Furthermore, labelling at Cα and Cβ positions can also be envisaged starting from commercially available labelled glycine using the Seebach's procedure reported for chiral imidazolidinone.[15]

2.2. Threonine incorporation

The incorporation of threonine in ubiquitin overexpressed in *E. coli* was further investigated. Addition of U-[^{13}C] threonine alone results in the labelling of the methyl groups of threonine in ubiquitin. As expected from the biosynthetic pathway, where isoleucine derives from threonine via the conversion of threonine to 2-oxobutanoate (Figure S2), we also observed the concomitant labelling of the methyl groups of Ile-δ_1 (Figure S3A). We then investigated the effect of addition of exogenous 2-oxobutanoate to inhibits scrambling of labelled Threonine in Isoleucine. Culture in presence of 150 mg/L of fully deuterated 2-oxobutanoate results in a complete absence of the signals from the methyl groups of Ile (Figure S3B). This is likely to be due to the inhibition of the isoleucine biosynthetic pathway at the level of the threonine dehydratase (TD) by Isoleucine produced from the exogenous 2-oxobutanoate, as well as a dilution of the remaining endogenous ^{13}C-labelled 2-oxobutanoate by the added perdeuterated precursor. As previously reported in the literature,[11] when U-[^{13}C]-threonine is added alone in the culture without any other deuterated compounds, a labelling is observed for the glycine, the ε-CH$_3$ of methionine and at a lower level on the β-methyl position of alanine. This scrambling significantly increases in presence of 2-oxobutanoate. This phenomenon is most likely due to the threonine degradation into glycine *via* the conjugate activities of threonine dehydrogenase and CoA ligase[16] or the threonine aldolase,[17] all enzymes being present in *E. coli*. The produced labelled glycine is further converted in serine, followed by deamination to produce pyruvate, the precursor for alanine synthesis. Glycine is also at the root of the one-carbon cycle, this induce labelling of the ε-CH$_3$ of methionine through this pathway. These pathways are very active as 30% of the ε-CH$_3$ of methionine are labelled in presence of threonine (Figure S3A). The scrambling can be diluted by adding 500 mg/L deuterated glycine. This last condition results in a complete absence of any labelling in these positions (data not shown). Interestingly, in the case of addition of threonine protonated and ^{13}C-labelled only on the Cγ methyl group, the glycine formed, arising from backbone atoms, is fully deuterated and not ^{13}C-labelled, and no scrambling in alanine, methionine can then be detected in the ^{13}C-HMQC spectra (Figure S3C).

Threonine incorporation was quantified using increasing amounts of threonine in the medium, either without or with the presence of perdeuterated 2-oxobutanoate at a concentration of 150 mg/L. By adding threonine at a concentration of about 200 mg/L and without 2-oxobutanoate, saturation of the threonine metabolic pathway was achieved (incorporation level > 95%) (Figure 2).

In presence of 2-oxobutanoate and deuterated glycine, the saturation is obtained with four time less labelled threonine
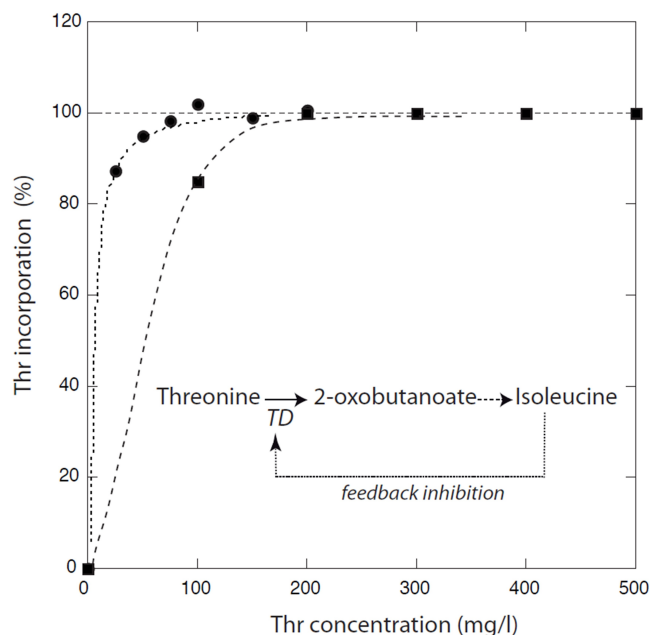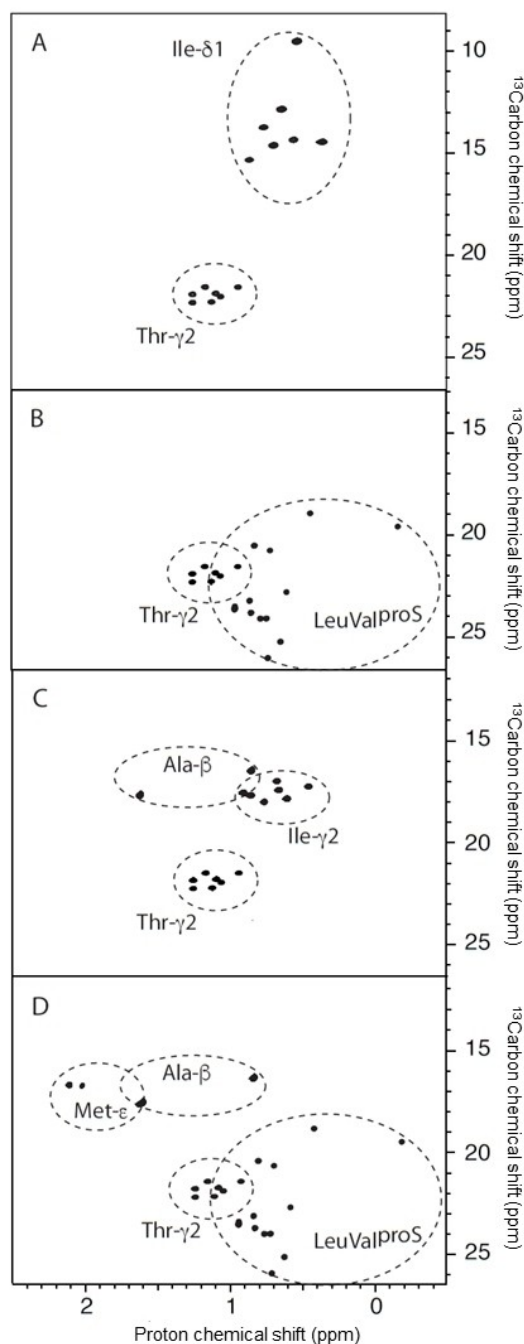

Figure 2. Incorporation of labelled *L*-threonine in overexpressed proteins. Incorporation level as a function of the concentration of threonine added alone 1 h prior induction (squares), or in presence of 150 mg/L of perdeuterated 2-oxobutanoate and 500 mg/L of deuterated glycine (circles) to inhibit metabolic scrambling into Alanine, Glycine, Isoleucine, and Methionine.

(i.e. 50 mg/L of threonine) compared to the quantity required to reach the same incorporation level without 2-oxobutanoate. This amount can be further reduced by a factor of two using auxotroph strains.[18]

2.3. Combinatorial labelling of threonine with other methyl containing amino acids

NMR spectra of ubiquitin labelled with synthesized threonine incorporated in presence of deuterated glycine and 2-oxobutanoate demonstrate that isotope enrichment is specific for Thr-γ_2 methyl sites without any unwanted labelling of other positions such as methine, methylene or other methyl sites. This result indicates that labelled *L*-threonine can be used with other known precursors to label any combination of methyl groups. For instance, [^{13}CH$_3$]-*L*-Thr, used in combination with the affordable precursor [^{13}CH$_3$]-2-oxobutanoate, allows the simultaneous labelling of the Ile-δ_1 and Thr-γ_2 methyl groups[11] in perdeuterated proteins without consuming a high quantity of the labelled *L*-Thr as previously discussed (Figure 3A).

It is also possible to use other combinations involving residues whose biosynthetic pathways are independent.[19] This is illustrated on 2D methyl TROSY spectra (Figure 3, acquired using ubiquitin labelled on various combinations of methyl groups: Thr-γ_2, and *pro-S* methyl groups of Leu-δ_2, Val-γ_2 (Figure 3B); Thr-γ_2, Ala-β, Ile-γ_2 methyl groups (Figure 3C) or Thr-γ_2, Met-ϵ, Ala-β, Leu-δ_2, Val-γ_2 (Figure 3D).
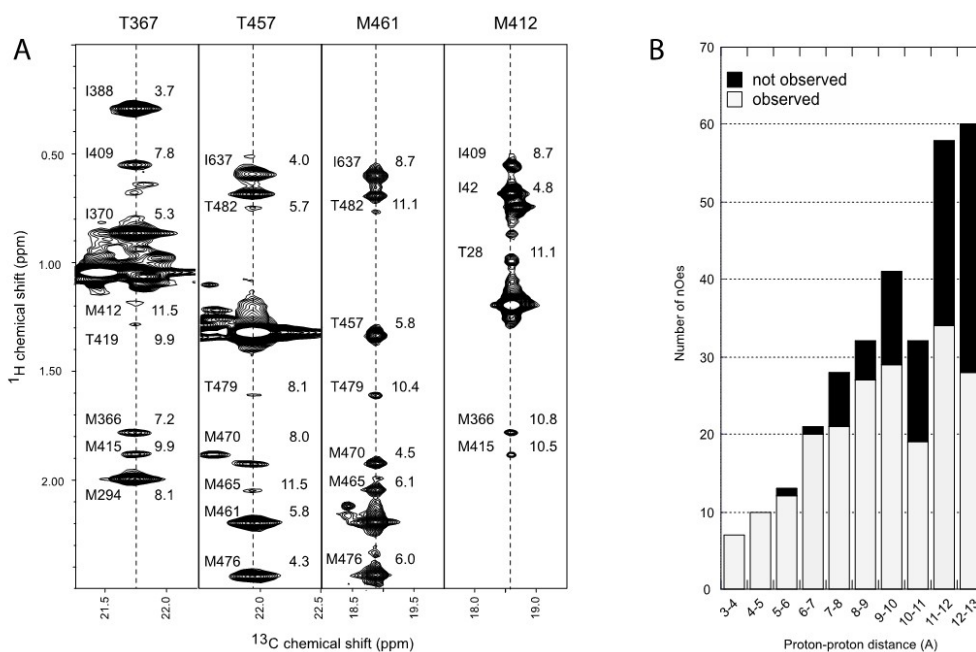
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Figure 3. Combinatorial methyl labelling. Extracts of the methyl region of 2D-^{13}C-HMQC spectra of ubiquitin produced in E. coli grown in a fully deuterated M9 medium (^{2}H$_2$O and 2 g/L of deuterated glucose) in presence of the following precursors added 1 h prior to IPTG induction. **A**: 50 mg/L 2,3-[^{2}H$_2$]- 4-[^{13}C]-L-threonine, 150 mg/L 2-oxo-3-[^{2}H$_2$]-4-[^{13}C]-butanoate and 500 mg/L of deuterated glycine; **B**: 50 mg/L 2,3-[^{2}H$_2$]-4-[^{13}C]-L-threonine, 300 mg/L 2-hydroxy-2-[^{13}C]-3-oxo-4-[^{2}H$_3$]-butanoate, 150 mg/L 2-oxo-3,4-[^{2}H$_5$]-butanoate and 500 mg/L deuterated glycine, **C**: 50 mg/L 2,3-[^{2}H$_2$]-4-[^{13}C]-L-threonine, 100 mg/L 2-hydroxy-2-[^{2}H$_5$]-ethyl-3-oxo-4-[^{13}C]-butanoate, 250 mg/L 2-[^{2}H]- 3-[^{13}C]-L-alanine, 200 mg/L deuterated 2-keto-isovalerate and 500 mg/L deuterated glycine, **D**: 50 mg/L 2,3-[^{2}H$_2$]- 4-[^{13}C]-L-threonine, 300 mg/L 2-hydroxy-2-[^{13}C]-3-oxo-4-[^{2}H$_3$]-butanoate, 250 mg/L 2-[^{2}H]-3-[^{13}C]-L-alanine, 100 mg/L (S)-2-hydroxy-2-[^{2}H$_5$]-ethyl-3-oxo-4-[^{2}H$_3$]-butanoate, 100 mg/L ε-^{13}C-methionine and 500 mg/L deuterated glycine.
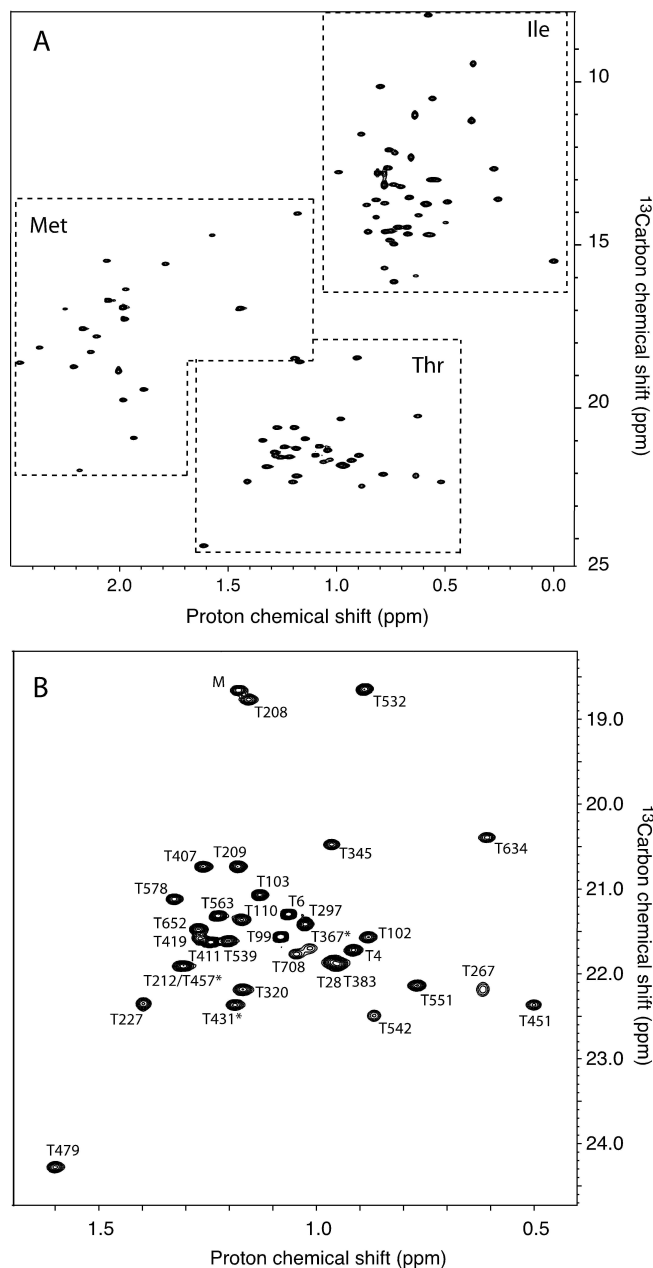
Figure 4. A: 2D-^{13}C-HMQC spectra of MSG produced in E. coli cells grown in a fully deuterated medium (^{2}H$_2$O and 2 g/L of deuterated glucose) in presence of 50 mg/L 2,3-[^{2}H$_2$]-4-[^{13}C]-L-threonine, 150 mg/L 2-oxo-3-[^{2}H$_2$]-4-[^{13}CH$_3$]-butanoate and 100 mg/L of ε-^{13}CH$_3$ deuterated methionine; the signals of γ$_2$-methyles of threonine, δ$_1$-methyls of isoleucine and ε-methyls of methionine are well separated as materialized by the dotted boxes. **B**: Zoom on the region of spectra corresponding to Thr-γ$_2$ correlations, annotated with corresponding residue numbers. Spectrum was recorded at 37 °C on a NMR spectrometer operating at a ^{1}H frequency of 800 MHz.

2.4. Detection of nOes between remote methyl probes into the 82 kDa Malate Synthase G

The benefits of labelling specifically combination of methyl groups can be clearly seen in NMR spectra of large perdeuterated proteins.[20] In a first study, we have shown that long-

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Figure 5. A: 2D extracts of the 3D-HMQC-NOESY recorded on perdeuterated MSG sample specifically $^{13}CH_3$-labelled on Ile-δ_1, Thr-γ_2, Met-ϵ positions. The 2D extracts are showing nOes connectivites used to assign T365, T457, M461, M412. Each strip was centered at the carbon chemical shift of the corresponding methyl. The assignments for the observed nOes are indicated along on the left and the corresponding distances (Å) for each pair of methyl group is indicated on the right. Several nOes arising from distances above 10 A could be observed. **B**: Distribution of the averaged inter-methyl ^1H-^1H distances in MSG at 37 °C, including number of detected nOes as a function of inter-methyl distances depicted with grey bars. Supplementary pairs of methyl groups, for which the nOes are not detected, are displayed with black bars. MSG X-ray structure[22] (PDB: 1D8 C) has been used as reference to calculated expected ^1H-^1H distance for each methyl pair.

range nOes between methyl groups which are up to 12 Å apart[21] in small proteins were detectable in 2D ^1H-^1H NOESY spectra. We have also shown that such intermethyl nOes remain detectable in perdeuterated malate synthase G (MSG; MW = 82 kDa) between methyl probes distant by 10 Å in 3D ^{13}C-edited HMQC-NOESY.[22]

In this experiment, the 33 Thr-γ, 44 Ile-δ_1 and 22 Met-ϵ methyl groups of MSG were selectively labelled. Indeed, these different types of methyl groups appear in distinct regions of the 2D methyl TROSY spectra, hence reducing the possibility of overlap. The 2D methyl-TROSY spectrum of U-[^2H, ^{12}C, ^{15}N], γ_2-[$^{13}CH_3$]-Thr, ϵ-[$^{13}CH_3$]-Met, δ_1-[$^{13}CH_3$]-Ile-labelled MSG, expressed in *E. coli* supplemented with 2,3-[$^2H_2$]-4-[$^{13}CH_3$]-threonine, ϵ-[$^{13}CH_3$] deuterated methionine and 2-oxo-3-[$^2H_2$]-4-[$^{13}CH_3$]-butanoate, is shown in figure 4A. These data confirm the high quality of the sample prepared and the exceptional selectivity of the labelling strategy.

Previous strategy based on the residual protonation obtained when proteins are overexpressed in M9/D$_2$O based medium using U-[^{13}C,^1H]-glucose as carbon source, was used to label and assign methyl groups of alanine and threonine residues of the Malate Synthase G.[23] In such sample proton density is significantly reduced and $^{13}CD_2H$ isotopomer was used exclusively. Such labelling scheme leads to a significant reduction of signal to noise ratio of corresponding NMR spectra compared to spectra acquired with sample enriched with $^{13}CH_3$-labelled threonine.

With the protocol described here, the protonation of methyl is complete and we can acquire 3D ^{13}C-edited NOESY spectra with a high signal to noise ratio[22,24] to complete assignment using the X-ray 3D structure of MSG (PDB:1D8 C) as reference.[25] We corroborated most of the reported assignments and were able to assign 3 extra threonine methyl resonance previously unassigned. (Table S1, Figure 4B, Figure S4). This structure based assignment strategy using nOes connectivities, allows us also to assign most of the ϵ-[$^{13}CH_3$]-Met of MSG (17 Met over 22, Table S2, Fig S4). Examples of 2D strips showing Thr and Met assignments together with long-range nOes are presented in Figure 5A. Full analysis of the 3D HMQC-NOESY spectrum indicated that 86 % of the expected nOes between the methyl pairs separated by 3–9 Å were detected (theoretical distances were predicted from the reference X-ray structure). Moreover, about 60 % of the nOes arising from methyl groups separated by 9–12 Å could also be detected. A histogram presenting the number of intermethyl nOes as a function of the average distance between both probes is presented in figure 5B. For such large protein, characterized by an overall tumbling of *c.a* 40 ns,[24] we can still detect 50 % of predicted nOes between methyl probe distant by 12 Å. However, to extract distance restraints in this highly deuterated protein, it is important to account for the contribution of spin diffusion since as a relatively long mixing period (400 ms) was used to detect a maximum number of long range nOes. Full relaxation-matrix analysis is required to extract

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accurate distance restraints from such 3D NOESY spectra.[21,26] Such long-range distance restraints are particularly useful for the structural investigation of large proteins.[27]

3. Conclusion

This research reports an efficient 6-steps protocol for the chemical synthesis of deuterated *L*-threonine specifically labelled with a $^{13}CH_3$ probe, using commercially available labelled synthetic intermediates. This synthesis scheme opens an alternative route to produce this important methyl containing amino acid and can be expanded to produce the *L*-threonine with different labelling schemes. The suitably labelled *L*-threonine can be incorporated in perdeuterated proteins overexpressed in *E. coli,* enabling NMR studies of high molecular weight proteins. We propose various protocols for combinatorial labelling of methyl probes of threonine together with other methyl containing amino-acids. Application of such protocols to the 82 kDa Malate Synthetase G demonstrates that optimal combinatorial labelling can be used to extract a large number of structurally meaningful distance restraints between remote methyl probes separated by up to 12 Å. We expect that this labelling strategy will complement existing protocols and will be particularly useful to investigate structure, dynamics, interaction and mechanism of large protein and complexes using NMR spectroscopy.

Supporting Information Summary

Detailed synthetic as well as experimental procedures are given in supporting information. Spectral data of organic compounds are also included.

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

Keywords: NMR · asymmetric synthesis · Threonine · Methyl · Isotopic labelling · Large proteins · Distance restraints · nOes

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