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Synthesis of ^{13}C-methyl-labeled amino acids and their incorporation into proteins in mammalian cells†

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Isotopic labeling of methyl-substituted proteinogenic amino acids with ^{13}C has transformed applications of solution-based NMR spectroscopy and allowed the study of much larger and more complex proteins than previously possible with ^{15}N labeling. Procedures are well-established for producing methyl-labeled proteins expressed in bacteria, with efficient incorporation of ^{13}C-methyl labeled metabolic precursors to enable the isotopic labeling of Ile, Val, and Leu methyl groups. Recently, similar methodology has been applied to enable ^{13}C-methyl labeling of Ile, Val, and Leu in yeast, extending the approach to proteins that do not readily fold when produced in bacteria. Mammalian or insect cells are nonetheless preferable for production of many human proteins, yet ^{13}C-methyl labeling using similar metabolic precursors is not feasible as these cells lack the requisite biosynthetic machinery. Herein, we report versatile and high-yielding synthetic routes to ^{13}C methyl-labeled amino acids based on palladium-catalyzed C(sp^3)–H functionalization. We demonstrate the efficient incorporation of two of the synthesized amino acids, ^{13}C-γ2-Ile and ^{13}C-γ1,γ2-Val, into human receptor extracellular domains with multiple disulfides using suspension-cultured HEK293 cells. Production costs are reasonable, even at moderate expression levels of 2–3 mg purified protein per liter of medium, and the method can be extended to label other methyl groups, such as ^{13}C-δ1-Ile and ^{13}C-δ1,δ2-Leu. In summary, we demonstrate the cost-effective production of methyl-labeled proteins in mammalian cells by incorporation of ^{13}C methyl-labeled amino acids generated *de novo* by a versatile synthetic route.

Introduction

Structural characterization of proteins by NMR has long relied on uniform labeling with ^{15}N and ^{13}C isotopes and assignment of signals based on transfer of spin coherence between connected backbone nuclei.[1,2] Subsequent transfer of spin coherence to side chain nuclei can enable nearly complete assignment of the ^{1}H, ^{13}C, and ^{15}N resonances and, in turn, full structure determination based on identification of inter-residue NOEs, residual dipolar couplings (RDCs), and other restraint types.[3–6] In spite of the success of this approach, and its extension to larger proteins by deuteration[7,8] and sensitivity enhancement by cross-correlation approaches,[9,10] this strategy is still limited for larger proteins due to the unfavorable transverse spin relaxation properties of backbone and most side-chain nuclei. In addition, this approach is only applicable to proteins that can be produced with uniform ^{15}N and ^{13}C labeling and thus it is practically restricted to proteins that can be produced in bacteria or methylotrophic yeast, such as *Pichia pastoris*.[11,12]

One method that has been impactful for overcoming challenges associated with unfavorable transverse spin relaxation of larger proteins is selective isotopic labeling of the sidechain methyl groups with ^{13}C.[13–16] Sidechain methyl groups have much more favorable transverse spin relaxation properties compared to backbone nuclei due to rapid (ps timescale) rotation around the three-fold methyl bond axis. Methyls also have other benefits for observation in larger proteins, including increased signal intensity due to the chemical equivalence of the three methyl protons and insensitivity to pH, enabling observation at higher pH values without accompanying loss of signal intensity due to solvent exchange. Robust procedures

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for selective isotopic labeling of the sidechain methyl groups of Ile, Leu, and Val with ^{13}C are well-established for proteins expressed in bacteria. Typically, this involves inclusion of simpler ^{13}C-methyl-labeled metabolic precursors, such as 3-^{13}C-3,4,4′,4″-^{2}H-α-ketoisovalerate for Leu-δ1,δ2 and Val-γ1,γ2 or 4-^{13}C-3,3′-^{2}H-α-ketobutyrate for Ile-δ1 in $^{2}H_2O$-based growth medium.[14–18] Procedures have also been developed for the backbone-independent assignment of methyl signals, the most reliable of which is mutagenesis,[19] though for larger proteins with many methyl groups this may be impractical, and other approaches that leverage existing structural information may be used to attain assignments.[20–28]

Methyl labeling, coupled with sensitivity enhancement by cross-correlation approaches, has had an enormous impact in terms of extending the applicability of NMR to study the structure and function of much larger and more complex systems than previously possible using uniform labeling with ^{15}N, ^{13}C, and ^{2}H isotopes.[14,16] Nonetheless, there are still many proteins, such as mammalian membrane receptors or secreted proteins that are heavily disulfide-bonded, that can be very difficult or impossible to express in bacteria, precluding methyl labeling using the aforementioned metabolic precursors. Recently, it was shown that yeast cultured on $^{2}H_2O$-containing medium with 4-^{13}C-α-ketobutyrate or 3-^{13}C-α-ketoisovalerate could be used to generate Ile-δ1, Val-γ1,γ2, or Leu-δ1,δ2 labeled proteins, extending the strategy of labeling methyls with low-cost metabolic precursors to proteins that were previously inaccessible by bacterial expression.[11,12]

Methyl labeling in yeast, however, is not without limitations – for example, the labeling efficiency is lower than that possible by bacterial expression (*ca.* 45% for Ile-δ1, 74% for Val-γ1,γ2, and 10% for Leu-δ1,δ2), and for both Val-γ1,γ2 and Leu-δ1,δ2, achieving this level of efficiency requires cultivation under acidic conditions, which many membrane receptors or other complex human proteins might not tolerate.[11,12] Moreover, many proteins are not correctly folded by the yeast protein production machinery; thus, mammalian cells, such as human embryonic kidney (HEK293) or Chinese hamster ovary (CHO) cells, remain the most suitable hosts for expression of mammalian proteins.[29] Mammalian cells also maintain native post-translational modifications that may be critical to produce functionally active proteins. However, due to the inability of mammalian cells to synthesize most of the methyl-bearing amino acids, including Ile, Leu, and Val, from simple building blocks,[30] it is not generally possible to generate methyl-labeled proteins in mammalian cells simply using the types of metabolic precursors described above.

One possible approach for producing ^{13}C-methyl labeled amino acids in mammalian cells is to use alternative metabolic precursors, such as ^{13}C uniformly labeled D-glucose, which can be used to label the methyl groups of alanine due to conversion of glucose to pyruvate and in turn alanine by aminotransferases.[31] However, it is not possible to label other methyl-bearing amino acids using this approach, nor is it possible to achieve near complete labeling of alanine by metabolic labeling from ^{13}C uniformly labeled D-glucose;[31] thus, alternative approaches are needed. One such approach is to synthesize the requisite amino acids with ^{13}C at the desired methyl positions and use these to construct medium that sustains the growth of CHO or HEK293 cells for protein expression. In the past, adoptions of this approach were limited due to low expression yields and the extended duration required to generate a stable transfected cell line that produced the target protein at high levels. However, advancements over the past two decades have significantly mitigated these challenges and newer expression systems based on modified versions of HEK293 or CHO have become an established method of choice for mammalian protein production.[32] Furthermore, the inclusion of an N-terminal signal peptide together with either a hexahistidine or strep tag in the protein's sequence allows for a swift one-step purification process directly from the conditioned medium.

Labeling of the methione methyl group (^{13}C-ε-Met) with high efficiency in mammalian cells is now relatively well established, which is not surprising, given the low cost of ^{13}C-ε-Met and the generally lower amounts of this amino acid required in the medium compared to most other amino acids. In the past several years, there are reports of successfully labeling the methyl groups of other amino acids, such as Val and Leu,[33,34] though this is not as widespread, presumably due to the high cost of these building blocks. Thus, although there has been progress towards producing ^{13}C-methyl labeled amino acids in mammalian cells, one barrier that remains is the availability of low-cost synthetic routes to the requisite ^{13}C methyl-labeled amino acids.

To address this challenge, we report here synthetic routes for ^{13}C-γ2-Ile·HCl (**1**) and ^{13}C-γ1,γ2-Val·HCl (**2**) using palladium-catalyzed C(sp^3)–H functionalizations.[35,36] The key step (s) were performed using an *N*-phthaloyl (NPhth) protected L-alanine (**3**) residue bearing an 8-aminoquinoline amide at the C-terminus as a directing auxiliary (DA) group. Iodomethane-^{13}C was used for ^{13}C-methyl-labeling. The introduction of the ^{13}C methyl-labeled group demonstrated a high degree of regio- and stereoselectivity at the β-position due to the DA coordination to the transition metal, resulting in the *anti*-configuration in the case of ^{13}C-γ2-Ile·HCl (**1**).[37] The critical steps of the syntheses of **1** and **2** included the prevention of a potential erosion of diastereoselectivity at the epimerizable stereogenic carbons during the removal of DA and NPhth groups. Therefore, mild conditions were developed for the deprotection steps, suitable for the required synthetic scale. Starting with 1 to 2 g of iodomethane-^{13}C, this synthetic route afforded 100 to 250 mg of the two target amino acids, ^{13}C-γ2-Ile·HCl (**1**) and ^{13}C-γ1,γ2-Val·HCl (**2**), and enabled the expression of 1 to 2 mg of two different 20 kDa disulfide-bonded receptors using suspension cultured HEK293 freestyle cells. The labeling efficiency of ^{13}C-γ2-Ile and ^{13}C-γ1,γ2-Val was found to be near 100% by comparison of the ^{13}C-γ2-Ile and ^{13}C-γ1,γ2-Val signal intensities relative to those in a natural abundance sample, and there was no apparent scrambling to other amino acids. Thus, we demonstrate the cost-effective and efficient production of ^{13}C methyl-labeled proteins in

mammalian cells, thereby expanding the benefits of methyl-labeling to larger and more challenging protein targets than previously feasible.

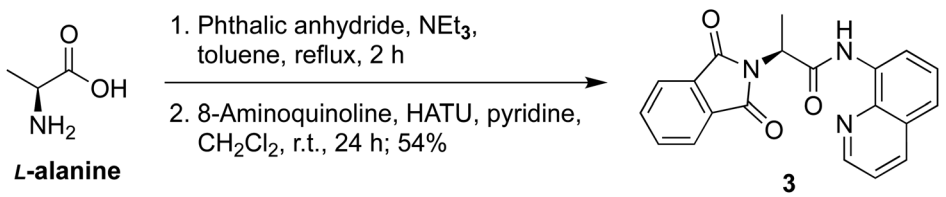
Results

Synthesis of ^{13}C methyl-labeled γ2-Ile and γ1,γ2-Val

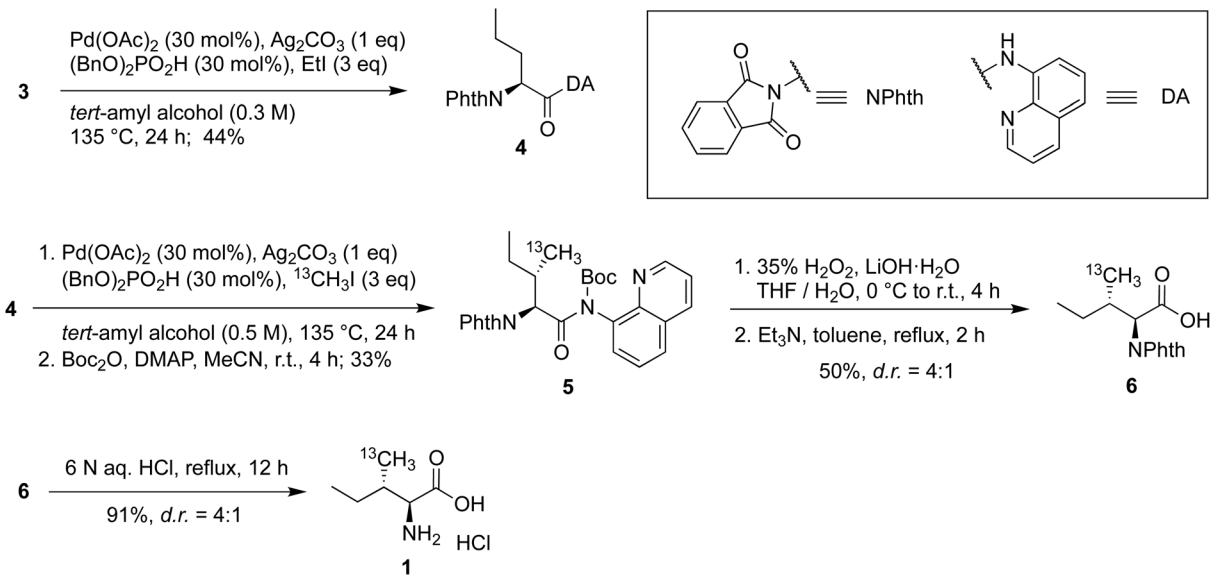
Both ^{13}C methyl-labeled γ2-Ile·HCl (**1**) and ^{13}C methyl-labeled γ1,γ2-Val·HCl (**2**) were obtained from the known intermediate **3**, which was synthesized from L-alanine by protecting the amine as *N*-phthaloyl (NPhth) imide followed by coupling of the resulting carboxylic acid with 8-aminoquinoline.[38–40] Intermediate **3** was obtained in 54% yield over two steps (Scheme 1).

For the synthesis of ^{13}C methyl-labelled γ2-Ile (**1**), we first performed a C(sp^3)–H functionalization on intermediate **3** using similar reaction conditions to our previously reported protocol,[41] which were obtained after an extensive optimization of literature conditions and scale-up,[38,42] leading to the generation of the 2-aminovaleric backbone **4** in 44% yield (Scheme 2). The second functionalization allowed us to introduce the ^{13}C methyl-group with the (*S*)-configuration in a good diastereomeric ratio and obtain the ^{13}C methyl-labeled γ2-Ile scaffold. At this point, we faced the critical step to selectively remove the DA and NPhth groups without affecting the two stereocenters. Therefore, compound **5** was synthesized by Boc-protection of the previous intermediate in 33% yield over two steps, allowing the subsequent cleavage of the amide bond under mild conditions.[43] Then, DA was removed using 35% H_2O_2 and LiOH, affording a mixture of the desired product and the carbamoylbenzoic acid by-product. The latter was converted into the desired phthalimide-protected analog **6** by treating the crude mixture with triethylamine in toluene at reflux in 50% yield, providing a 4 : 1 ratio of diastereomers. The deprotection of the phthalimide group of **6** was performed using well-established conditions to preserve the configuration at the α-stereocenter, affording ^{13}C methyl-labeled γ2-Ile·HCl **1** in 91% yield.

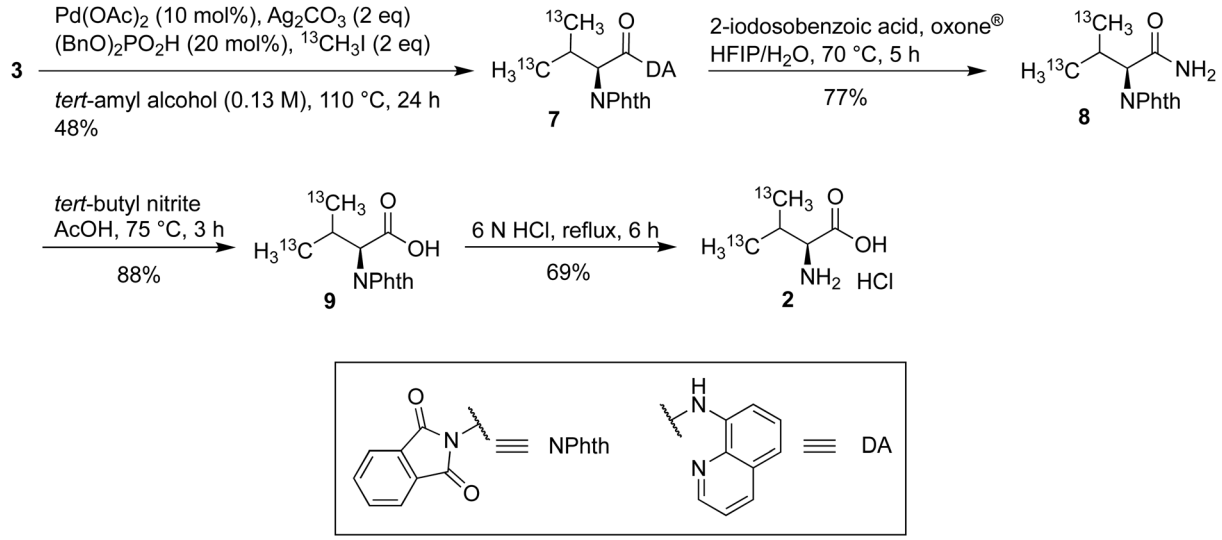
The synthesis of ^{13}C methyl-labeled γ1,γ2-Val (**2**) commenced with the C(sp^3)–H functionalization of **3** using iodomethane-^{13}C to give the ^{13}C methyl-labeled γ1,γ2-Val scaffold **7** in 48% yield (Scheme 3). The 8-aminoquinoline amide was converted into the primary amide **8** in 77% yield using 2-iodosobenzoic acid and oxone® and then into the corresponding carboxylic acid **9** using *tert*-butyl nitrite in acetic acid in 88% yield. These deprotection conditions were chosen to facilitate the purification of intermediates, and, sim-



Scheme 1 Synthesis of key intermediate **3**.



Scheme 2 Synthesis of ^{13}C methyl-labeled γ2-Ile·HCl (**1**).



Scheme 3 Synthesis of methyl-^{13}C labeled γ1,γ2-Val·HCl (**2**).

ultaneously, avoid erosion of stereoselectivity.[43,44] In the past several years, successful labeling of the methyl groups of other amino acids, such as Val, has been reported.[31,45] The ^{13}C methyl-labeled γ1,γ2-Val·HCl (**2**) was obtained by heating **9** at reflux in a 6 N HCl solution for 6 h.

Protein labelling

In this study, we choose as a model two related proteins: the C-terminal zona pellucida domain of the TGF-β co-receptor betaglycan, BG_{ZPC},[46,47] and a pituary-specific paralog known as TGFBRIII-like or R3like. The zona pellucida domain is a small (~20 kDa) extracellular domain with 3 to 4 disulfide bonds that is present in proteins involved in various biological processes, including cell signaling and cell adhesion. The betaglycan ZPC domain is responsible for binding to the signaling proteins TGF-β and inhibinA and modulating signaling outcomes, whereas R3like binds and modulates signaling of inhibinB.[48–50] Dysregulation of these interactions may contribute to the development of inflammatory disorders, cancer, or infertility. Despite significant efforts, structures of these receptor extracellular domains, which both contain three disulfide bonds, bound to their cognate signaling ligands are not known and thus our efforts are directed towards using NMR approaches to determine the structures of the corresponding ligand–receptor complexes. Although BG_{ZPC} can be produced in bacteria in form of inclusion bodies and refolded to a natively folded protein, the folding efficiency is poor and the production, refolding, and purification is complicated and lengthy, making it an expensive and difficult protein to produce using bacterial expression. R3like can also be produced in bacteria in the form of inclusion bodies, but we have been unable to obtain natively folded protein by refolding. BG_{ZPC} and R3like represent a common outcome where expression of disulfide-rich mammalian proteins can be difficult to obtain using the *E. coli* expression system. On the other hand, both proteins can be natively folded by HEK293 cells and secreted into the medium, from which they can be easily collected and purified using a tag-based affinity purification.

BG_{ZPC} ^{13}C-γ2-Ile and ^{13}C-γ1,γ2-Val labelling

We have previously established a robust protocol for producing BG_{ZPC} in suspension cultured HEK293F cells,[51] with a yield of approximately 4 mg of fully purified protein per liter of commercially available complete Hyclone TransFx-H liquid medium. We therefore adapted this procedure to produce methyl-labeled BG_{ZPC} since the production yield is reasonable and since this same growth medium is commercially available without amino acids. We showed through pilot studies that the production yield with medium constructed with amounts of unlabeled amino acids detailed in the Methods section afforded an overall yield of protein that was reduced by only a factor of 2 relative to commercially available complete medium. We therefore proceeded to produce BG_{ZPC}, with labeling of either synthesized ^{13}C-γ2-Ile and commercially available ^{13}C-ε-Met (BG_{ZPC} IM) or synthesized ^{13}C-γ1,γ2-Val and commercially available ^{13}C-ε-Met (BG_{ZPC} VM). In addition, since this protein can be also produced with some effort in *E. coli*, we decided to use this to our advantage to compare the ^{13}C linewidths of the valine methyl ^{13}C NMR signals from the incorporated amino acids to those obtained from the established bacterial ILVM method for ^{13}C-methyl labeling with background deuteration. Notably, in both cases, we enriched the medium with a saturating concentration of ^{13}C-ε-Met to serve as a reference for complete (100%) labeling.

With the amounts of ^{13}C-γ2-Ile and ^{13}C-γ1,γ2-Val synthesized from 1–2 g scale quantities of the input low cost ^{13}C reagent, iodomethane-^{13}C (Table 1, upper portion), and with the production yields and medium composition noted above, we were able to readily produce sufficient quantities of fully

Table 1 Amino acid synthesis and protein production yields

AA	Amount $^{13}CH_3I$ (g)	Amount AA synthesized (mg)
^{13}C-γ2-Ile	2.29	236
^{13}C-γ1,γ2-Val	1.14	78

Protein sample	Amount AA (mg)	Volume medium (mL)	Protein amount (mg)
BG_{ZPC} ^{13}C-γ2-Ile	100	500	1.1
BG_{ZPC} ^{13}C-γ1,γ2-Val	78	420	0.8
R3like ^{13}C-γ2-Ile	100	500	0.7

purified BG_{ZPC} IM and BG_{ZPC} VM for several 300 μL NMR samples at a concentration of 50 μM (Table 1, lower portion). We further observed that the spectra of the labeled proteins yielded signals only in the chemical shift ranges expected for the type of label incorporated and that the number of signals observed was in accord with the number expected based on the amino acid sequence (13 Val, 6 Ile, and 5 Met), both for the control ^{13}C-ε-Met and the synthesized ^{13}C-γ2-Ile or ^{13}C-γ1,γ2-Val (Fig. 1A–D). We also observe that the majority of the peak positions in the labeled samples match the signals in the much more concentrated natural abundance sample (Fig. 1E and F). Minor chemical shift differences visible on the spectra may originate from a small propensity to self-associate at the higher concentration, but not lower concentrations, variation in the glycosylation of the BG_{ZPC} domain at one of the two possible N-linked glycosylation sites that it possesses, or small difference in buffer conditions.

Production of secreted receptors in mammalian cells typically results in significant mass heterogeneity, due to heterogenous glycosylation. Production in glycosylation-deficient cell lines and aggressive treatment with deglycosidases, such as PNGaseF, can result in samples that approach the expected mass of the core protein and are more mass homogenous, yet it is nonetheless difficult to obtain a completely homogenous sample that can be used to accurately assess the incorporation efficiency of ^{13}C- and ^{15}N-labeled amino acids using mass spectrometry. Hence, as an alternative approach, we compared the relative signal intensities of ^{13}C-ε-Met/^{13}C-γ1,γ2-Val and ^{13}C-ε-Met/^{13}C-γ2-Ile in the BG_{ZPC} VM and BG_{ZPC} IM samples that we prepared relative to the same signals in a sample of BG_{ZPC} at natural abundance (Fig. 2A and B). Incorporation of ^{13}C-ε-methionine into protein derived from mammalian cultures has also been shown to occur with an efficiency of 95% or greater.[52] If so, this suggests that incorporation of both ^{13}C-γ1,γ2-Val and ^{13}C-γ2-Ile using our HEK293F expression system occurs with comparable efficiency based on the near equal Met/Val and Met/Ile signal intensity ratios relative those of the BG_{ZPC} natural abundance sample (Fig. 2A and B).

Selectively protonating ^{13}C-methyl groups within a highly deuterated environment using the *E. coli* system has the advantage of reducing the dipolar relaxation contribution from neighboring protons. In accord with this, we observe that the ^{13}C line widths for the ^{13}C-γ1,γ2-Val signals in the *E. coli* produced deuterated ILVM sample (ESI, Fig. 1S†), which are generally more rigid and thus more susceptible to effects from dipolar broadening compared to that of the more flexible Met-ε signals, are on average only 1.2-fold greater than those of the corresponding ^{13}C-ε-Met signals (Fig. 2C). In contrast, the ^{13}C line widths for the ^{13}C-γ1,γ2-Val signals in the mammalian produced protonated VM sample are roughly a factor of 1.6 greater than those of the corresponding ^{13}C-ε-Met signals (Fig. 2C). Owing to the less crowded nature of ^{1}H–^{13}C methyl correlation spectra compared to ^{1}H–^{15}N amide correlation spectra, adverse consequences due to resonance overlap are reduced, enabling the clear and distinct discrimination of signals. Nonetheless, in the context of large proteins or protein complexes, it would be advisable to explore modifications in amino acid synthesis to facilitate the incorporation of deuterium in positions adjacent to the ^{13}C-methyl site, both to reduce potential overlap and to increase sensitivity by sharpening the signals as recently shown for the incorporation of ^{13}C-δ2-Leu in otherwise protonated background.[45]

R3like ^{13}C-γ2-Ile labelling

Human R3like proved to be difficult to refold from reconstituted bacterially expressed inclusion bodies, despite significant structural similarity to the BG_{ZPC} domain as predicted by AlphaFold and a preserved number of internal disulfides. However, production and purification of R3like from HEK293 cell culture could be successfully accomplished,[48] and using the same labeling strategy demonstrated for BG_{ZPC}, we succeeded in obtaining a sufficient quantity of ^{13}C-ε-Met/^{13}C-γ2-Ile R3like for several 50 μM NMR samples from 100 mg of ^{13}C-γ2-Ile (Table 1, lower portion). Similar to that observed for BG_{ZPC}, we observe signals only in the chemical shift ranges expected for the incorporated labels and the number of observed signals matches expectations (4 Ile and 1 Met) (Fig. 3). Chemical shifts recorded for ^{13}C-γ2-Ile suggest that the protein sample is natively folded.

Discussion

Methyl-labeling has greatly extended the applicability of NMR to much larger and much more complex systems than what was possible using traditional approaches based upon observation and assignment of backbone ^{15}N and ^{13}C signals. However, methyl-labeling in mammalian cells is not possible

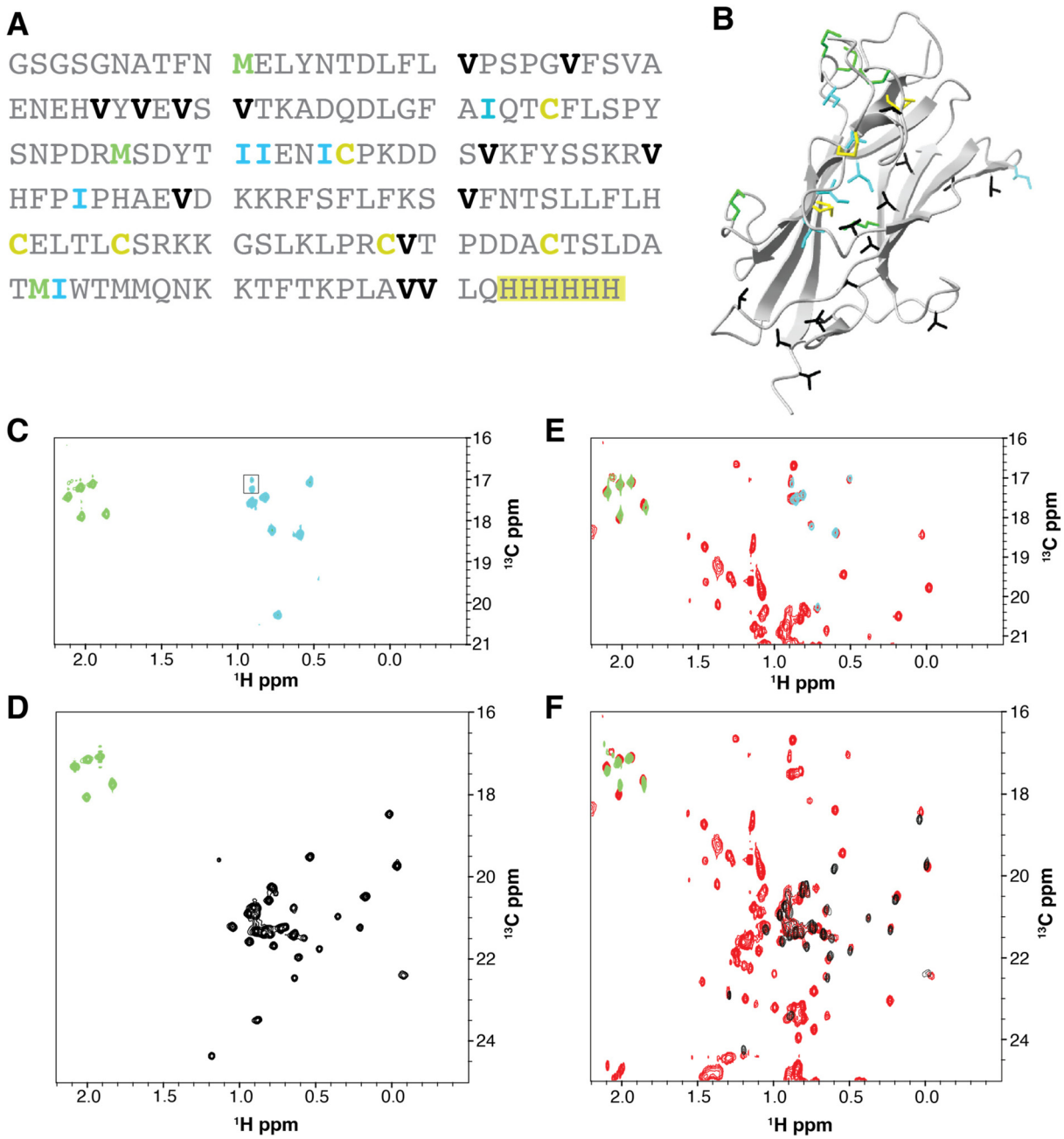


Fig. 1 ^{13}C-Methyl-labeling of the Ile-γ2, Val-γ1,γ2, and Met-ε signals of BG_{ZPC} as expressed in suspension-cultured HEK293F cells. (A). Amino acid sequence of BG_{ZPC}. Ile, Val, and Met residues are highlighted in cyan, black, and green, respectively. The artificial C-terminal histidine tag used for purification is highlighted in yellow. (B). Structure of BG_{ZPC} (PDB 3QW9) highlighting the locations of its three disulfide bonds (yellow) and the Ile, Val, and Met residues (cyan, black, and green, respectively). (C) and (D). ^{1}H–^{13}C HSQC shift correlation spectrum of BG_{ZPC} labeled with ^{13}C-γ2-Ile and ^{13}C-ε-Met (C) or ^{13}C-γ1,γ2-Val and ^{13}C-ε-Met (D). Signals originating from ^{13}C-γ2-Ile, ^{13}C-γ1,γ2-Val, and ^{13}C-ε-Met are shaded cyan, black, and green, respectively. (E) and (F). Reference ^{1}H–^{13}C HSQC spectra of BG_{ZPC} at natural abundance as produced using mammalian cell expression. HSQC spectra for ^{13}C-γ2-Ile and ^{13}C-ε-Met and ^{13}C-γ1,γ2-Val and ^{13}C-ε-Met labeled BG_{ZPC} (same spectra as shown in panels C and D, respectively) are overlaid on top of the reference ^{1}H–^{13}C HSQC spectrum (red) of BG_{ZPC} at natural abundance (E and F, respectively).

using metabolic precursors, as it is widely done in bacteria and increasingly in yeast, due to the absence of the requisite biosynthetic machinery, though as recently shown uniformly ^{13}C-labeled D-glucose can be used to label alanine methyl groups with 25% efficiency.[31]

Herein, we report an efficient synthetic route to ^{13}C-labeled amino acids based on the palladium-catalyzed C(sp^3)–H functionalization of protected L-alanine and demonstrate its effectiveness through the synthesis of ^{13}C-γ2-Ile and ^{13}C-γ1,γ2-Val. We demonstrate that these amino acids can be incorpor-

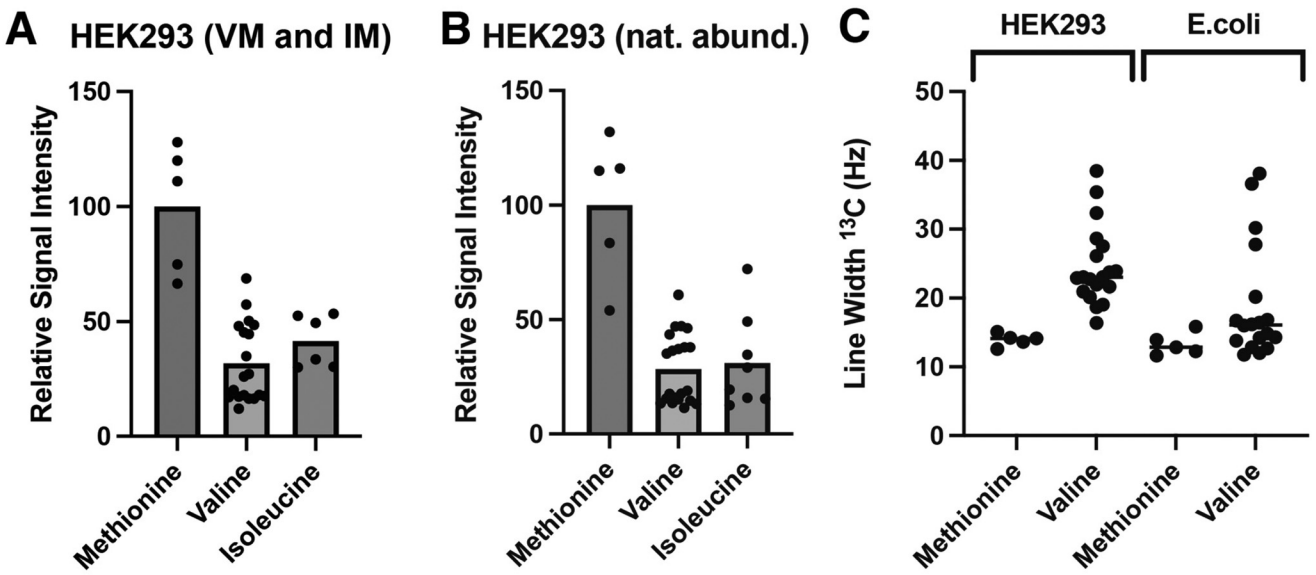


Fig. 2 Incorporation efficiency and linewidths for methyl-labeling in mammalian cells. (A) and (B). Estimation of incorporation levels of ^{13}C-γ2-Ile and ^{13}C-γ1,γ2-Val by comparison of their intensities, relative to that of ^{13}C-ε-Met, in either a sample of BG_{ZPC} produced in mammalian cells with ^{13}C-γ2-Ile/^{13}C-ε-Met or ^{13}C-γ1,γ2-Val/^{13}C-ε-Met labeling (A) or at natural abundance (B). Column bars represent the mean values of peak volumes as measured in the CCpNMR Analysis Software package. (C). Comparison of ^{13}C line widths in Hertz of Val-γ1,γ2 methyl peaks in relation to Met-ε methyl peaks in protein samples obtained from bacterial or mammalian cell culture. The increased linewidths for methyl ^{13}C-valine signals, but not the methyl ^{13}C-methionine signals, is consistent with the valine methyls generally being more rigid and thus sensitive to whether the neighbouring carbons are deuterated or not, while the comparable linewidths for the methione methyls suggest these are more flexible and less sensitive to the level of deuteration.

ated into two different disulfide bonded receptor extracellular domains using suspension cultured HEK293 cells transiently transfected with the plasmid coding for the protein of interest. We observe by comparison of signal intensities to those in a natural abundance sample that the efficiency of labeling is near 100%. We also demonstrate there is little to no scrambling to other amino acids and that labeling is cost-efficient, with an input of 1 to 2 g of $^{13}CH_3I$ being adequate to generate sufficient ^{13}C-methyl labeled amino acids to produce several 300 μL batches of 50 μM NMR samples. Importantly, the establishment of this low-cost and simple technology extends methyl-labeling approaches to many large and complex human proteins that cannot be easily produced in a native functional state by either bacterial or yeast expression.

For some applications, such as the determination of complex structures based on observation of inter-molecular inter-methyl NOEs, it may be important to achieve a higher density of signals than is possible with labeling of only ^{13}C-γ2-Ile and ^{13}C-γ1,γ2-Val. In light of this, it will be important to extend the synthetic methodology that we have developed to generate other types of ^{13}C-methyl-labeled amino acids, such as ^{13}C-δ1,δ2-Leu and ^{13}C-γ1-Val. Owing to the versatile nature of the synthesis that we have developed, it may be possible to attain ^{13}C-δ1,δ2-Leu by performing the C(sp^3)–H functionalization of intermediate **3** using 2-iodopropane-1,3-$^{13}C_2$ instead of iodomethane-^{13}C, or by using a γ-activating DA instead of the β-activating DA 8-aminoquinoline amide.[53] ^{13}C-γ1-Val can be obtained by a mono-functionalization of **3** using iodomethane-^{13}C followed by a second C–H functionalization with unlabelled CH_3I. Furthermore, it is feasible to attain other ^{13}C-methyl-labeled amino acids, such as phenylalanine, tyrosine and tryptophan by using the corresponding ^{13}C-labeled electrophiles iodobenzene, 4-iodophenol, and N-protected iodoindole, respectively.[42]

As it was demonstrated, in the absence of uniform deuteration, the linewidths for ^{13}C-labeled methyl groups appeared broader compared to the highly deuterated background used in *E. coli* production. Alternative approaches will be required to attenuate transverse relaxation through dipolar coupling to other protons. One possible approach to attenuate relaxation, as recently shown for incorporation of ^{13}C-δ2-Leu into an IgG,[45] is to modify the synthesis to replace protons immediately adjacent to the ^{13}C-methyl with deuterons. In the case of ^{13}C-γ2-Ile and^{13}C-γ1,γ2-Val this could possibly be done by palladium-catalyzed hydrogen/deuterium exchange on the NH Boc-deprotected intermediate of compound **5** and intermediate **7** in the presence of D_2O, respectively.[54]

A further extension of the synthetic approach that we have developed is the synthesis of amino acids with fluorinated methyl groups, and the incorporation of these into proteins using bacterial, yeast, or mammalian cell expression systems. Due to the high sensitivity for detection of ^{19}F, and the absence of background ^{19}F signals in biological systems, this approach offers significant promise for studying protein structure and dynamics in living cells, as recently demonstrated by

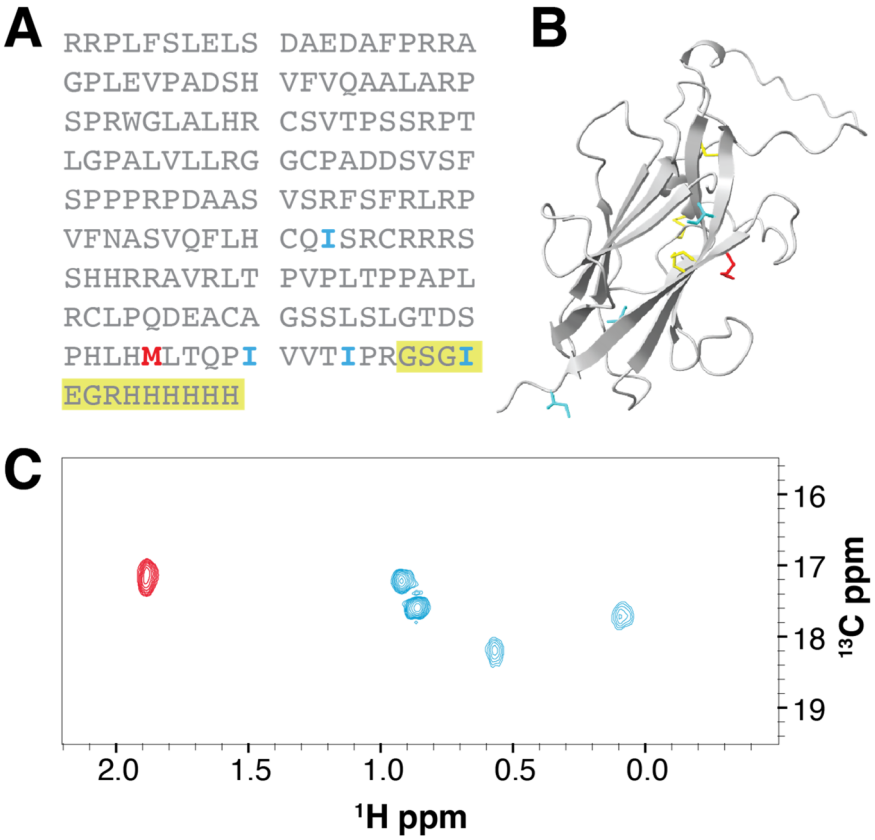


Fig. 3 ^{13}C-Methyl-labeling of the Ile-γ2 and Met-ε signals of R3like as expressed in suspension-cultured HEK293 (HEK293F) cells. (A). Amino acid sequence of R3like. Ile and Met residues are highlighted in cyan and red, respectively. The artificial C-terminal factor Xa cleavage site and the histidine tag used for processing and purification are highlighted in yellow. (B). Alphafold2 structure of R3like highlighting the locations of its three disulfide bonds (yellow) and the Ile and Met residues (cyan and red, respectively). (C). ^{1}H–^{13}C HSQC shift correlation spectrum of R3like labeled with ^{13}C-γ2-Ile and ^{13}C-ε-Met. Signals originating from ^{13}C-γ2-Ile and ^{13}C-ε-Met are shaded red and cyan, respectively.

the successful detection of ^{19}F-Trp- and ^{19}F-Tyr-labeled proteins incorporated into mammalian cells.[55]

Experimental

General methods for chemical synthesis and analysis

Unless stated otherwise, all reactions were performed under an atmosphere of N_2 that was passed through a column (10 × 2 cm) of Drierite®. Degassed solvents were prepared by freeze–pump–thaw techniques. All glassware and stir bars were flame-dried or stored in a drying oven prior to use. Reactions were monitored by TLC (thin layer chromatography) analysis using pre-coated silica gel 60 F254, and spots were visualized using UV light at 254 nm and 385 nm. Purifications by chromatography were performed on SiO_2. ^{1}H/^{13}C NMR spectra were recorded on Bruker Avance 300/76 MHz, Bruker Avance 400/101 MHz, Bruker Avance 500/126 MHz or Bruker Avance 601/151 MHz instruments. High resolution mass spectra were obtained on a Micromass UK Limited, Q-TOF Ultima API or a Thermo Scientific Exactive Orbitrap LC-MS. IR spectra were obtained using neat samples on a PerkinElmer 100 IR ATR spectrometer. Melting points were obtained using a Mel-Temp instrument and are not corrected. Chemical shifts were reported in parts per million (ppm) with the residual solvent peak ($CDCl_3$: 7.26 ppm for ^{1}H, 77.16 ppm for ^{13}C; DMSO-d_6: 2.50 ppm for ^{1}H, 39.52 ppm for ^{13}C) used as the internal standard. Chemical shifts were tabulated as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, h = sextet, dd = doublet of doublet, dt = doublet of triplet, ddd = doublet of doublet of doublet, tt = triplet of triplet, dtd = double of triplet of doublet, dddt = doublet of doublet of doublet of triplet, ddq = doublet of doublet of quartet, dtt = doublet of doublet of triplet, tqd = triplet of quartet of doublet, dpd = doubled of pentet of doubled, tdd = triplet of doublet of doublet, qdd = quartet of doublet of doublet, m = multiplet, br s = broad singlet), coupling constant(s), and integration.

(*S*)-2-(1,3-Dioxoisoindolin-2-yl)-*N*-(quinolin-8-yl)propenamide (3)

A solution of phthalic anhydride (4.16 g, 28.1 mmol), L-alanine (2.50 g, 28.1 mmol, 1 eq.), and triethylamine (1.18 mL, 8.42 mmol, 0.3 eq.) in toluene (22 mL, 1.3 M) was heated at reflux for 2 h. The reaction mixture was cooled to room temp-

erature and concentrated *in vacuo*. The crude residue was acidified by addition of 1 N aq. HCl (40 mL) and extracted with CH_2Cl_2 (2×). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated to give crude (*S*)-2-(1,3-dioxoisoindolin-2-yl)propanoic acid as a white solid that was used in the next step without further purification.

A solution of (*S*)-2-(1,3-dioxoisoindolin-2-yl)propanoic acid (4.90 g, 22.4 mmol, 1 eq.) in CH_2Cl_2 (20 mL, 1.1 M) was treated sequentially with 8-aminoquinoline (2.53 g, 17.2 mmol, 0.77 eq.), pyridine (2.85 mL, 34.6 mmol, 1.55 eq.), and HATU (8.50 g, 22.4 mmol, 1 eq.). The reaction mixture was stirred at ambient temperature for 24 h. The deep brown solution was diluted with ethyl acetate, washed with sat. $NaHCO_3$ and brine, dried ($MgSO_4$), and purified by chromatography on SiO_2 (2% acetone in CH_2Cl_2) to afford **3** (4.20 g, 12.2 mmol, 54%) as a white solid. Characterization data were in agreement with literature results.[41] According to this experimental protocol, Wang *et al.* obtained **3** with an enantiomeric ratio (er) of 99.8 : 0.2.[39]

(*S*)-2-(1,3-Dioxoisoindolin-2-yl)-*N*-(quinolin-8-yl)pentanamide (4)

A mixture of amide **3** (0.50 g, 1.45 mmol, 1 eq.), $Pd(OAc)_2$ (98 mg, 0.43 mmol, 30 mol%), Ag_2CO_3 (0.40 g, 1.45 mmol, 1 eq.), $(BnO)_2PO_2H$ (123 mg, 0.434 mmol, 30 mol%), and ethyl iodide (356 μL, 4.34 mmol, 3 eq.) in *tert*-amyl alcohol (4.4 mL, 0.3 M) in a microwave glass vial (purged with argon and sealed with a PTFE cap) was heated at 135 °C for 24 h. The reaction mixture was filtered through a pad of Celite and concentrated *in vacuo*. The crude residue was purified by chromatography on SiO_2 (25% acetone in hexanes) to afford **4** (0.24 g, 0.63 mmol, 44%) as a white solid. Characterization data were in agreement with literature results:[38] **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 10.35 (s, 1 H; quinoline N=C<u>H</u>–), 8.80–8.51 (m, 2 H; quinoline signals), 8.15 (dd, J = 8.3, 1.7 Hz, 1 H; quinoline signals), 7.90 (dt, J = 6.9, 3.5 Hz, 2 H; NPhth –CH=C<u>H</u>–C), 7.76 (dd, J = 5.5, 3.1 Hz, 2 H; NPhth –C<u>H</u>=CH–C), 7.51 (dd, J = 4.2, 3.1 Hz, 2 H; quinoline signals), 7.43 (dd, J = 8.3, 4.3 Hz, 1 H; quinoline signals), 5.15 (dd, J = 11.1, 5.2 Hz, 1 H; –C<u>H</u>CON–), 2.67–2.54 (m, 1 H; –C<u>H</u>$_2$CH–), 2.46–2.30 (m, 1 H; –C<u>H</u>$_2$CH–), 1.47 (h, J = 7.4 Hz, 2 H; –C<u>H</u>$_2$CH$_3$), 1.04 (t, J = 7.4 Hz, 3 H; –CH$_2$C<u>H</u>$_3$).

tert-Butyl ((2*S*,3*S*)-2-(1,3-dioxoisoindolin-2-yl)-3-(methyl-^{13}C) pentanoyl)(quinolin-8-yl)carbamate (5)

A mixture of amide **4** (1.51 g, 4.05 mmol, 1 eq.), $Pd(OAc)_2$ (273 mg, 1.21 mmol, 30 mol%), Ag_2CO_3 (1.12 g, 4.05 mmol, 1 eq.), $(BnO)_2PO_2H$ (345 mg, 1.21 mmol, 30 mol%), and iodomethane-^{13}C (753 μL, 12.1 mmol, 3 eq.) in *tert*-amyl alcohol (7.5 mL, 0.5 M) in a microwave glass vial (purged with argon and sealed with a PTFE cap) was heated at 135 °C for 24 h. The reaction mixture was filtered through a pad of Celite® (CH_2Cl_2 to rinse, 250 mL collection flask), and concentrated *in vacuo*. The crude residue was re-suspended in a mixture of 50% acetone/hexanes, filtered through a pad of SiO_2 (50% acetone in hexanes to rinse, 500 mL collection flask) and concentrated *in vacuo*. The resulting crude mixture containing the desired intermediate, bis-alkylated byproduct, and remaining starting material was purified by reverse-phase chromatography (C18, 30 g cartridge, H_2O/MeOH; 6 CV of 100% water, then 80 CV of gradient elution to 100% MeOH) to remove the remaining starting material. The resulting mixture of desired intermediate and bis-alkylated byproduct was used for the next step without further purification.

A solution of this mixture (0.88 g, 2.26 mmol, 1 eq.) Boc_2O (1.48 g, 6.79 mmol, 3 eq.), and DMAP (0.55 g, 4.53 mmol, 2 eq.) in anhydrous CH_3CN (5 mL, 0.4 M) was stirred at room temperature for 4 h, concentrated *in vacuo* and purified by chromatography on SiO_2 (10% acetone in hexanes) to afford **5** (1.90 g, 3.96 mmol, 33% yield over two steps) as a white foam: **IR** (ATR, CH_2Cl_2) 3061, 2933, 2971, 2876, 1740, 1716, 1500, 1469, 1369, 1293, 1265, 1254, 1153, 1125 cm^{-1}; **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 8.62 (br s, 1 H; quinoline signal), 8.10 (dd, J = 8.2, 1.8 Hz, 1 H; quinoline signal), 7.85 (dd, J = 5.5, 2.9 Hz, 2 H; NPhth –CH=C<u>H</u>–C), 7.77 (dd, J = 8.3, 1.4 Hz, 1 H; quinoline signal), 7.76–7.67 (m, 3 H; quinoline signal overlapped with NPhth –C<u>H</u>=CH–C), 7.54 (t, J = 7.8 Hz, 1 H; quinoline signal), 7.30 (td, J = 8.4, 4.1 Hz, 1 H; quinoline signal), 5.93–5.63 (m, 1 H; –C<u>H</u>CON–), 2.73–2.58 (m, 1 H; –C<u>H</u>^{13}CH$_3$), 1.57 (dpd, J = 14.5, 7.3, 3.3 Hz, 1 H; –C<u>H</u>$_2$CH$_3$), 1.29 (d, J = 6.7 Hz, 1.5 H; –CH^{13}C<u>H</u>$_3$), 1.17–0.93 (m, 11.5 H; –C(C<u>H</u>$_3$)$_3$ overlapped with –C<u>H</u>$_2$CH$_3$ and –CH^{13}C<u>H</u>$_3$), 0.88 (t, J = 7.4 Hz, 3 H; –CH$_2$C<u>H</u>$_3$); **^{13}C NMR** (151 MHz, Chloroform-*d*) δ 171.9, 167.8, 152.7, 150.1, 134.1, 132.0, 129.2, 128.9, 128.1, 126.6, 123.5, 121.5, 83.0, 58.8, 27.4, 25.6, 23.1, 22.9, 17.0, 15.7, 11.8, 11.7, 11.5; **HRMS** (ESI$^+$) m/z calcd for $C_{27}{}^{13}CH_{30}O_5N_3$ [M + H]$^+$, 489.2214; found, 489.2198.

(2*S*,3*S*)-2-(1,3-Dioxoisoindolin-2-yl)-3-(methyl-^{13}C)pentanoic acid (6)

A solution of the Boc-protected intermediate **5** (1.80 g, 3.69 mmol, 1 eq.) in THF and water (3 : 1, THF/H_2O, 7.2 mL, 0.5 M) was cooled to 0 °C and treated with 30% hydrogen peroxide (3.26 mL, 32.5 mmol, 8.8 eq.) and lithium hydroxide monohydrate (233 mg, 5.54 mmol, 1.5 eq.), stirred at 0 °C for 45 min, warmed to room temperature and stirred for an additional 3 h. The reaction mixture was quenched at 0 °C with 1.5 M aqueous sodium thiosulfate (2 mL) and concentrated under reduced pressure. The residue was washed with CH_2Cl_2 (2×), and the aqueous phase was then acidified to pH 1–2 with 10% aqueous HCl and extracted with EtOAc (2×). The combined organic extracts were dried (Na_2SO_4) and concentrated under reduced pressure. Both product and the ring-opened phthalimide byproduct were present and the mixture was directly used in the next step. A suspension of the crude mixture (0.27 g, 0.97 mmol, 1 eq.) and triethylamine (0.07 mL, 0.48 mmol, 0.5 eq.) in toluene (3 mL) was stirred for 2 h at reflux, cooled to room temperature and concentrated *in vacuo*. The crude residue was acidified by addition of 10% aqueous HCl and extracted with EtOAc (2×), dried (Na_2SO_4), filtered, and concentrated *in vacuo*. Purification by chromatography on SiO_2 (2% MeOH in CH_2Cl_2) gave **6** as a white solid (0.42 g,

1.61 mmol, 50% yield over two steps in *trans*/*cis* = 4 : 1 diastereomeric ratio): **^{1}H NMR** (400 MHz, Chloroform-*d*, 60 °C) Major diastereomer: δ 7.88 (ddd, J = 5.4, 3.1, 1.1 Hz, 2 H; NPhth –CH=CH–C), 7.74 (ddd, J = 5.5, 3.1, 1.1 Hz, 2 H; NPhth –CH=CH–C), 4.73 (ddd, J = 8.6, 3.4, 1.1 Hz, 1 H; –CHCO$_2$H–), 2.64–2.50 (m, 1 H; –CH^{13}CH$_3$), 1.53 (dtd, J = 13.6, 6.9, 4.1 Hz, 1 H; –CH$_2$CH$_3$), 1.32 (d, J = 6.7 Hz, 1.5 H; –CH^{13}CH$_3$), 1.20–1.07 (m, 1 H; –CH$_2$CH$_3$), 1.04–0.97 (m, 1.5 H; –CH^{13}CH$_3$), 0.89 (td, J = 7.4, 1.1 Hz, 3 H; –CH$_2$CH$_3$); **^{13}C NMR** (151 MHz, Chloroform-*d*) Major diastereomer: δ 173.6, 168.0, 134.5, 131.7, 123.8, 57.3, 34.5 (d, J = 35.3 Hz), 22.5 (d, J = 34.8 Hz), 16.9, 10.9 (d, J = 35.0 Hz).

γ^{I}-(^{13}C)-L-Isoleucine·HCl (1)

A solution of **6** (0.40 g, 1.53 mmol, 1 eq.) in 6 N aq. HCl (20 mL) was heated at reflux for 12 h in a pressure tube. The precipitated phthalic acid was removed by extraction with AcOEt (3×). The water layer was freeze-dried to give **1** (236 mg, 1.40 mmol, 91%, in *trans*/*cis* = 4 : 1 diastereomeric ratio) as a white solid: α_{25}^{D} +5.1 (c 0.1, H$_2$O); **^{1}H NMR** (500 MHz, DMSO-d_6) Major diastereomer δ 13.74 (s, 1 H; –CO$_2$H), 8.37 (s, 3 H, –NH$_3^+$), 3.79 (s, 1 H, CHCO$_2$H), 1.92 (tdd, J = 9.1, 5.8, 3.1 Hz, 1 H; –CH(^{13}CH$_3$)), 1.48 (qdd, J = 12.9, 10.4, 6.5 Hz, 1 H; –CH$_2$CH$_3$), 1.35–1.23 (m, 1 H; –CH$_2$CH$_3$), 1.05 (d, J = 6.9 Hz, 1.5 H; –^{13}CH$_3$), 0.89 (td, J = 7.4, 4.4 Hz, 3 H; –CH$_3$), 0.80 (d, J = 6.9 Hz, 1.5 H; –^{13}CH$_3$); **^{13}C NMR** (126 MHz, DMSO-d_6) Major diastereomer: δ 170.1 (d, J = 2.3 Hz), 56.0, 35.6 (d, J = 34.8 Hz), 21.6 (d, J = 34.7 Hz), 14.4, 11.5 (d, J = 2.2 Hz); **HRMS** (ESI$^+$) m/z calcd for C$_5$^{13}CH$_{12}$O$_2$N, 131.0896, found 131.0898.

(*S*)-2-(1,3-Dioxoisoindolin-2-yl)-3-(methyl-^{13}C)-*N*-(quinolin-8-yl) butanamide-4-^{13}C (7)

A mixture of **3** (0.70 g, 2.03 mmol, 1 eq.), Pd(OAc)$_2$ (50 mg, 0.20 mmol, 10 mol%), silver carbonate (1.13 g, 4.05 mmol, 2 eq.), (BnO)$_2$PO$_2$H (115 mg, 0.40 mmol, 20 mol%) was added to a flame-dried 75 mL pressure tube. Then, *tert*-amyl alcohol (15.6 mL, 0.13 M) was added followed by iodomethane-^{13}C (0.25 mL, 4.05 mmol, 2 eq.). The reaction mixture was heated at 110 °C for 24 h, diluted with CH$_2$Cl$_2$, and then filtered through a pad of Celite® (CH$_2$Cl$_2$ to rinse), and concentrated *in vacuo*. The crude residue contained the desired product along with the mono-alkylated intermediate and the starting material and was re-subjected to the same reaction conditions to push the reaction to completion. After the work-up, the crude residue was purified by chromatography on SiO$_2$ (10% acetone in hexanes) to give **7** (0.37 g, 0.98 mmol, 48%) as a pale-yellow solid: **^{1}H NMR** (400 MHz, Chloroform-*d*) δ 10.58 (s, 1 H; –CONH–), 8.85 (dd, J = 4.2, 1.7 Hz, 1 H; quinoline signal), 8.76 (dd, J = 5.0, 4.1 Hz, 1 H, quinoline signal), 8.14 (dd, J = 8.3, 1.7 Hz, 1 H, quinoline signal), 7.94–7.85 (m, 2 H; NPhth –CH=CH–C), 7.79–7.69 (m, 2 H; NPhth –CH=CH–C), 7.55–7.48 (m, 2 H; quinoline signal), 7.44 (dd, J = 8.3, 4.2 Hz, 1 H; quinoline signal), 4.70 (dt, J = 10.8, 2.8 Hz, 1 H; –CHCONH–), 3.23 (dddt, J = 13.3, 10.2, 6.6, 3.3 Hz, 1 H; –CH(^{13}CH$_3$)$_2$), 1.39 (dd, J = 6.7, 5.3 Hz, 1.5 H; –CH^{13}CH$_3$), 1.15 (dd, J = 6.6, 5.2 Hz, 1.5 H; –CH^{13}CH$_3$), 1.07 (dd, J = 6.6, 5.3 Hz, 1.5 H; –CH^{13}CH$_3$), 0.84 (dd, J = 6.6, 5.2 Hz, 1.5 H; –CH^{13}CH$_3$); **^{13}C NMR** (101 MHz, Chloroform-*d*) δ 168.3, 167.0, 148.7, 138.9, 136.3, 134.4, 134.4, 131.8, 128.1, 127.4, 123.8, 122.1, 121.8, 117.1, 63.4, 27.0 (d, J = 35.0 Hz), 20.6, 19.8, 15.8, 11.3 (dd, J = 35.1, 1.6 Hz).

(*S*)-2-(1,3-Dioxoisoindolin-2-yl)-3-(methyl-^{13}C)butanamide-4-^{13}C (8)

A 50 mL round-bottom flask was charged with **7** (369 mg, 0.99 mmol, 1 eq.), 2-iodosobenzoic acid (78.3 mg, 0.30 mmol, 30 mol%), and oxone® (1.82 g, 2.96 mmol, 3 eq.), followed by a mixture of HFIP/H$_2$O (1 : 1, 7.2 mL, 0.14 M). The reaction mixture was stirred at 70 °C for 5 h, quenched by the addition of sat. aq. NaHCO$_3$ and extracted with CH$_2$Cl$_2$ (3×). The combined organic layers were concentrated *in vacuo* and the crude residue was purified by chromatography on SiO$_2$ (30% hexanes in ethyl acetate) to give **8** (0.19 g, 0.76 mmol, 77%) as a white solid: **^{1}H NMR** (500 MHz, DMSO-d_6) δ 7.92–7.84 (m, 4 H; NPhth signals), 7.47 (s, 1 H; –CONH$_2$), 7.09 (s, 1 H; –CONH$_2$), 4.27 (dt, J = 8.6, 3.3 Hz, 1 H; –CHCONH$_2$), 2.73–2.61 (m, 1 H; –CH(^{13}CH$_3$)$_2$), 1.14 (dd, J = 6.7, 5.3 Hz, 1.5 H; –CH^{13}CH$_3$), 0.89 (ddd, J = 6.6, 5.1, 1.4 Hz, 3 H; –CH^{13}CH$_3$), 0.64 (dd, J = 6.8, 5.2 Hz, 1.5 H; –CH^{13}CH$_3$); **^{13}C NMR** (126 MHz, DMSO-d_6) δ 169.4, 167.7, 134.5, 131.3, 123.1, 58.5, 26.9 (d, J = 34.8 Hz), 20.9, 19.3, 15.6 (d, J = 1.9 Hz), 11.2 (dd, J = 34.9, 2.0 Hz).

(*S*)-2-(1,3-Dioxoisoindolin-2-yl)-3-(methyl-^{13}C)butanoic-4-^{13}C acid (9)

A stirred solution of the primary amide **8** (185 mg, 0.75 mmol, 1 eq.) in acetic acid (4 mL, 0.19 M) was dropwise treated with *tert*-butyl nitrite (149 μL, 1.13 mmol, 1.5 eq.) and stirred at 75 °C under air atmosphere for 3 h. The reaction progress was monitored by TLC. AcOH was removed by gently heating the flask to 40 °C while under high vacuum and cooling the receiver flask at −78 °C. The resulting residue was purified by chromatography on SiO$_2$ (2% MeOH in CH$_2$Cl$_2$) to afford **9** (0.16 g, 0.66 mmol, 88%) as a white solid: **^{1}H NMR** (300 MHz, Chloroform-*d*) δ 7.88 (dd, J = 5.5, 3.1 Hz, 2 H; NPhth –CH=CH–C), 7.75 (dd, J = 5.5, 3.0 Hz, 2 H; NPhth –CH=CH–C), 4.63 (dt, J = 8.5, 3.3 Hz, 1 H; –CHCO$_2$H), 2.86–2.67 (m, 1 H; –CH(^{13}CH$_3$)$_2$), 1.38 (dd, J = 6.6, 5.3 Hz, 1.5 H; –CH(^{13}CH$_3$)$_2$), 1.13 (dd, J = 6.8, 5.2 Hz, 1.5 H; –CH(^{13}CH$_3$)$_2$), 0.96 (dd, J = 6.6, 5.3 Hz, 1.5 H; –CH(^{13}CH$_3$)$_2$), 0.71 (dd, J = 6.8, 5.2 Hz, 1.5 H; –CH(^{13}CH$_3$)$_2$); **^{13}C NMR** (76 MHz, Chloroform-*d*) δ 173.0, 167.9, 134.5, 131.8, 123.8, 57.9, 27.3 (d, J = 34.9 Hz), 21.0, 19.6, 16.0 (d, J = 1.9 Hz), 11.4 (dd, J = 34.9, 1.9 Hz).

(2*S*)-2-Amino-3-(^{13}C)methyl(^{13}C)butanoic acid·HCl (2)

A solution of **9** (0.18 g, 0.73 mmol, 1 eq.) in 6 N aq. HCl (2.1 mL, 0.35 M) was heated at reflux for 6 h in a pressure tube. The precipitated phthalic acid was removed by extraction with AcOEt (3×). The water layer was freeze-dried to give **2** (78 mg, 0.50 mmol, 69%): α_{25}^{D} +47.8 (c 0.12, H$_2$O); **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.36 (br s, 3 H; –NH$_3^+$), 7.58–7.10 (m, 1 H; –CO$_2$H), 3.69 (q, J = 4.2 Hz, 1 H; –CHCO$_2$H), 2.19 (ddq, J = 11.9, 9.1, 4.7, 3.7 Hz, 1 H; –CH(^{13}CH$_3$)$_2$), 1.10 (td, J = 7.0, 4.9

Hz, 3 H; $-^{13}CH_3$), 0.85 (td, J = 6.9, 4.9 Hz, 3 H; $-^{13}CH_3$); **^{13}C NMR** (126 MHz, DMSO-d_6) δ 170.1, 57.4, 24.5 (d, J = 34.9 Hz), 18.1, 17.7, 14.3 (d, J = 2.2 Hz), 11.4 (dd, J = 34.9, 2.1 Hz); **HRMS** (ESI^+) m/z calcd for $C_3{}^{13}C_2H_{10}O_2N$, 118.0773, found 118.0776.

Mammalian plasmid expression constructs

Rat betaglycan ZPC domain (BG_{ZPC})[46,47] was expressed in freestyle suspension cultured HEK293 (HEK293F) cells (Invitrogen R79007) downstream of the rat serum albumin signal peptide and rat serum albumin and a thrombin cleavage site in pcDNA3.1+ (Invitrogen V79020). Mouse TGFβR3-like (R3like)[48] was expressed similarly, but downstream of the rat serum albumin signal peptide alone. Hexahistidine tags were appended to the C-terminal of both BG_{ZPC} and R3like to enable facile purification. Coding sequences of BG_{ZPC} and R3like were obtained by gene synthesis (Twist Biosciences) and the full coding cassettes were inserted between the NheI and XhoI sites in pcDNA3.1+. Constructs were verified by Sanger DNA sequencing over the entire length of the coding sequence.

Bacterial expression constructs

Rat betaglycan ZPC domain (BG_{ZPC}) was expressed in *E. coli* BL21(DE3) cells (EMD-Millipore 69450) downstream of thioredoxin, a hexahistidine tag, and a thrombin cleavage site in plasmid pET32a (EMD-Millipore 69015). The coding sequence for BG_{ZPC} was comprised of residues 590–757 of NCBI NP_058952 and a strepII tag, WSHPQFEK, was fused to its C-terminus. The coding sequence was obtained by gene synthesis (Twist Biosciences) and inserted between a KpnI site, which was introduced immediately following the last codon of the Trx-thrombin coding casette intrinsic to pET32a, and the XhoI site. Constructs were verified by Sanger DNA sequencing over the entire length of the coding sequence.

Protein expression and isotope labeling in mammalian cells

HEK293 freestyle cells (293F) were maintained in a custom formulation of HyClone HyCell TransFx-H medium (Cytiva). The custom formulated medium was prepared by mixing (per liter): 9.2 g TransFx-H media purchased without glucose or amino acids, 6 g glucose (Millipore-Sigma G7021), 3.2 g sodium bicarbonate (Millipore-Sigma S5761), 1 g Pluronic F-68 (AppliChem Panreac A12880500), 586 mg L-glutamine (Millipore-Sigma G8540), 50 mg L-alanine (Millipore-Sigma A7469), 500 mg L-arginine (Millipore-Sigma A8094), 900 mg L-asparagine (Millipore-Sigma A4159), 200 mg L-aspartic acid (Millipore-Sigma A7219), 100 mg L-cysteine-HCl (Millipore-Sigma C7477), 300 mg L-glutamic acid (Millipore-Sigma G8415), 30 mg glycine (Millipore-Sigma G5417), 200 mg L-histidine-HCl (Millipore-Sigma H6034), 400 mg L-isoleucine (Alfa Aesar J63045), 500 mg L-leucine (Millipore-Sigma L6914), 500 mg L-lysine-HCl (Millipore-Sigma L8662), 115 mg L-methionine (Millipore-Sigma M5308), 200 mg L-phenylalanine (Millipore-Sigma P8740), 500 mg L-proline (Millipore-Sigma P5607), 500 mg L-serine (Millipore-Sigma S4311), 380 mg L-threonine (Millipore-Sigma T8441), 200 mg L-tryptophan (Millipore-Sigma T8941), 200 mg L-tyrosine disodium dihydrate (J.T. Baker 2094-05), and 370 mg L-valine (Millipore-Sigma V0513). A suspension culture was grown on an orbital shaker (125 rpm) in a 37 °C incubator with 8% CO_2. Cells were passaged every 3–4 days by pelleting (8 min, 90*g*, 25 °C) and resuspending in fresh medium with a final density of 500 000 cells per mL.

In preparation for transfection to produce ^{13}C-γ2-Ile, ^{13}C-ε-Met (IM) or ^{13}C-γ1,γ2-Val, ^{13}C-ε-Met (VM) labeled Alb-BG_{ZPC} or R3like, cells were pelleted and resuspended in fresh media at 2 000 000 mL^{-1} in 50% of the desired volume for protein expression, termed the transfection volume, and allowed to continue to grow overnight. Three hours prior to transfection, the cells were pelleted and resuspended in fresh media, lacking the desired amino acid(s) to be labeled (dropout media), at 80% of the transfection volume using a density that would equate to 2 500 000 cells per mL for the transfection volume. Thirty minutes prior to transfection, the plasmid DNA (1.5 mg L^{-1} of transfection volume) and PEI (4.5 mg L^{-1} transfection volume, Polysciences 24765) were diluted in separate tubes with dropout media to 1 : 20. The PEI was added to the plasmid DNA and incubated at room temperature for 20 min. The DNA/PEI complex was then added along with media containing the labeled amino acids and an additional 4 mM L-glutamine to the cell suspension. Cells were allowed to grow overnight (14 h) before being diluted 2-fold with media containing the labeled amino acids, an additional 4 mM L-glutamine and valproic acid (1 mg mL^{-1}, Alfa Aesar A12962-18). The transfected cells were allowed to grow for 4–5 days before the conditioned media was harvested. Synthesized methyl-labeled amino acids, ^{13}C-δ1-Ile and ^{13}C-γ1,γ2-Val, were used at half the concentration of corresponding unlabeled amino acids. ^{13}C-ε-Met was used at the full concentration. Unlabeled Alb-BG_{ZPC} was produced using a similar procedure, but with expi293 suspension cultured HEK293 cells (Invitrogen A14527) and expi293 medium (Invitrogen A1435101), as previously described.[56]

Protein expression and isotope labeling in *E. coli*

Trx-BG_{ZPC} fusion protein isotopically labeled with ^{13}C-δ1-Ile, ^{13}C-γ1,γ2-Val, ^{13}C-δ1,δ2-Leu, and ^{13}C-ε-Met (ILVM) was produced by inoculating colonies from freshly transformed BL21 (DE3) cells into water-based M9 medium and incubated at 37 °C with shaking until the cell density reached OD_{600} 0.2–0.3. The cells were pelleted and resuspended in an equal volume of M9 medium that contained 99.9% ^2H_2O (Cambridge Isotope Laboratories DLM-4) and incubated at 37 °C with shaking until the cell density reached OD_{600} 0.6–0.65. Afterward, per liter of medium, 75 mg 4-^{13}C-3,3′-^{2}H-α-ketobutyric acid (Cambridge Isotope Laboratories CDLM-7318), 200 mg 3-^{13}C-3,4,4′,4″-^{2}H-α-ketovaleric acid (Cambridge Isotope Laboratories CDLM-7317), and 50 mg ^{13}C-ε-methionine (Cambridge Isotope Laboratories CLM-206) were added. Protein expression was induced 1 h later by adding 200 mg solid IPTG (Gold Bio, I2481) per 1 L of culture. Cells were harvested 6 h post induction by centrifugation.

Purification of BG_{ZPC} and R3like from mammalian cells

Conditioned media containing secreted protein was diluted 1.5× with buffer A (25 mM Na_2HPO_4, 150 mM NaCl, 6 mM imidazole, 1 mM $NiSO_4$ pH 8.0) and loaded onto a previously equilibrated HisPur NiNTA column (Thermo A50584). Protein was eluted with imidazole gradient 6 mM–500 mM over 10 column volumes (CV). Fractions containing albumin-BG_{ZPC} or R3like were pooled together and dialyzed against 20 mM HEPES, 150 mM NaCl, and concentrated. To separate BG_{ZPC} from albumin, 3 units of thrombin per mg of protein was added and the mixture was incubated with stirring overnight at 4 °C. The released BG_{ZPC}, and R3like, were further purified on a Superdex 75 26/60 size exclusion chromotography column (Cytiva 28-9893-34) equilibrated with dialysis buffer.

Purification of BG_{ZPC} from *E. coli*

Bacterial pellet from 6 liters culture of overexpressed Trx-BG_{ZPC} was resuspended in 300 mL of disruption buffer (100 mM Tris-HCl, 1 mM EDTA, pH 8.3) and sonicated in 50 mL batches (2.5 min, 500 W, 50% power, 50% duty cycle) on ice and centrifuged. The pellet, which contained the Trx-BG_{ZPC} as inclusion bodies, was washed sequentially, first by resuspending them in disruption buffer containing 0.5 M NaCl and then disruption buffer containing 1% Trition X-100. Inclusion bodies were then solubilized overnight in 200 mL 8 M urea, 25 mM Tris, 1 mM EDTA, reduced by addition of 30 mM DTT for 30 min, and acidified with glacial acetic acid to pH 4.5. Mixture was concentrated ten-fold, dialyzed against 8 M urea, 25 mM sodium acetate pH 4.5 and added dropwise to folding buffer (0.1 M Tris, 0.25 M guanidine HCl, 0.5 M L-arginine, 2 mM reduced glutathione (Millipore-Sigma G4251), 0.5 mM oxidized glutathione (Millipore-Sigma G4501), pH 8.0) at a protein concentration of 0.1 mg mL^{-1}. After stirring for 36 h at 4 °C, the folding mixture was concentrated, dialyzed against 0.1 M Tris, 0.15 M L-arginine pH 8.0, and BG_{ZPC} was cleaved from thioredoxin with 3U of thrombin per mg of fusion protein overnight at 4 °C with stirring. The digestion mixture was dialyzed against 25 mM sodium acetate pH 4.2 and diluted 1 : 1 with 8 M urea. This mixture was loaded onto a 1 cm × 8 cm Source S cation exchange column (Cytiva 17094410) equilibrated in 25 mM sodium acetate, 4 M urea, pH 4.2 and eluted with a linear salt gradient (0–1 M NaCl over ten column volumes). Fractions containing natively folded BG_{ZPC}, as assessed by NMR ^1H–^{15}N HSQC spectra, were further purified from mixture using streptactin-XT column (IBA Lifesciences 2-5030-002).

Protein NMR spectra

Samples of ^{13}C-methyl labeled BG_{ZPC} or R3like were dialyzed into 25 mM phosphate, 25 mM NaCl, pH 7.0, concentrated to a final protein concentration between 20–50 μM, and after adding ^2H_2O to 5%, samples were transferred to 5 mm susceptibility-matched NMR microtubes (Millipore-Sigma Z529451). A sample of unlabeled BG_{ZPC} was prepared similarly, but at a concentration of 700 μM. All NMR spectra were recorded using a Bruker AVII 800 MHz instrument equipped with a cryogenically cooled $^1H\{^{13}C,^{15}N\}$ 5 mm Z-gradient TCI cryoprobe, except for the BG_{ZPC} ILVM spectrum which was recorded with a similarly configured Bruker AVII 900 MHz instrument. All spectra were recorded with a ^1H–^{13}C HSQC pulse sequence with a WATERGATE solvent suppression scheme with 100 complex points and sweep width of 3623 Hz in the ^{13}C-dimension (36.2 Hz per point), except for the BG_{ZPC} ILVM spectrum which was recorded with 113 complex points and sweep width of 4075 Hz in the in the ^{13}C-dimension (36.2 Hz per point). All datasets were processed using NMRPipe[57] and analyzed with the CcpNmr analysis software package.[58]

Conflicts of interest

There are no conflicts to declare.

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