Synthesis of Isotopically Labelled, Spin-Isolated Tyrosine and Phenylalanine for Protein NMR Applications

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Author Contributions
B.M.Y. and P.J.S. carried out synthetic chemistry. B.M.Y. and Z.R. designed the study. P.R., Y.C., M.S., J.D., and C.G.K performed protein expression and protein NMR experiments. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website.
Complete synthetic details and characterization data of all novel compounds; NMR experimental data.

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Abstract

Isotopically labelled amino acids are widely used to study structure and dynamics of proteins by NMR. Herein we describe a facile, gram scale synthesis of compounds \(1b\) and \(2b\) under standard laboratory conditions from common intermediate \(7\). \(2b\) is obtained via simple deprotection while \(1b\) is accessed through a reductive deoxygenation/deuteration sequence and deprotection. \(1b\) and \(2b\) provide improved signal intensity using lower amounts of labelled precursor and are an alternative to existing labelling approaches.

Graphical Abstract

Protocols for the isotopic labelling of highly deuterated proteins to enable study by NMR are well established.\(^1\) Of recent interest, the ability to produce proteins containing isotopically labelled, spin-isolated aromatic acids have provided enhanced structural detail and enabled mechanistic study of protein kinases.\(^2\) In protein kinases, the knowledge of the Phe ring orientation in the conserved Asp-Phe-Gly motif (DFG) in solution is of great interest as it correlates to the active vs. inactive form.\(^3\) The isotope pattern specificity and high levels of incorporation necessary for the success of this method are achieved via introduction of advanced metabolic precursors that are transformed into the desired amino acid during protein expression \textit{in situ}.\(^4\) Phenylalanine \(1a\) and tyrosine \(2a\) are accessed via pyruvates \(3\) and \(4\) prepared from simple, commercially available isotopic building blocks and assembled in such a way that the desired isotope pattern is under complete synthetic control.\(^5\)

In the course of preparing proteins incorporating spin isolated aromatic amino acids, literature reported pyruvates \(3\) and \(4\) were synthesized in house and several challenges were noted. The synthesis of \(3\) and \(4\) diverge from common intermediate \(5\) at an early stage. The synthesis of \(3\) takes seven steps from \(5\) and requires manipulation and purification of four volatile intermediates. Preparation of \(4\) from \(5\) is carried out in six steps of which the final reaction requires rigorous exclusion of oxygen to prevent the product degradation. For the same reason, pyruvate \(4\) requires storage at \(-80^\circ\text{C}\), which presents an additional barrier to its use.\(^6\) In addition, up to 200 mg of \(3\) and \(4\) per liter of culture may be needed to achieve high levels of label incorporation into the protein for NMR studies using existing protocols.\(^4,\)\(^7\) In our hands, following the recently reported protocol for aromatic labelling using stereo-array isotope labeling (SAIL) amino acids,\(^2,\)\(^\text{c}\) we found \(3\) led to Phe \(1a\) incorporation at high levels (>90%) in the expressed protein at concentrations of 50 mg/mL (see Supporting Information, Figure 2). In contrast, total incorporation of \(2a\) remained ~7 fold lower than \(1a\) despite the use of increasing concentrations of \(4\) in the expression medium (Figure 2a).
Similar experience was reported by others, which led us to consider an alternative strategy for the introduction of 1a and 2a into our labelling experiments.

While unsure of the root cause of the low incorporation, we hypothesized that incorporating amino acids 1a and 2a directly, rather than their precursors, may increase labelling efficiency. Before initiating synthesis, we set the following criteria that we believed were critical to the design of an optimal synthetic approach to the target molecules: 1) identify a key intermediate suitable for providing both final products to minimize the number overall synthetic steps required, 2) eliminate handling and purification of volatile intermediates, and 3) utilize existing intermediates from the synthesis of 3 and 4 when possible.

With these goals in mind, we began our retrosynthetic analysis with a regioselective reductive deuteration/deoxygenation. That would allow production of 6 directly from 7, addressing our desire for a single advanced intermediate capable of providing both 1 and 2 (Scheme 1). However, this transformation had no direct examples in the literature. We anticipated that the access to 7 could be accomplished by Negishi cross coupling of iodoalanine 8 with iodophenol 9. Preparation of 9 by Sandmeyer iodination of 10, an intermediate in the production of 4, would allow access to desired labelling chemistry. Though use of 8 would produce isotopologues 1b and 2b with benzylic (Hβ) 1H in place of 2H, this change improves overall synthetic viability and maintains spin-isolation while facilitating aromatic assignment by providing a probe to connect intraresidue amide and Ce via 13C-edited and 15N-edited NOESY experiments (Figure 2b).

Before attempting the isotopically labelled synthesis, optimal conditions for the key reductive deuteration/deoxygenation of 7 were required. A survey of the literature revealed no examples of the desired transformation utilizing a deuterium source, although several examples of reductive deoxygenation of Tyr or its derivatives with a proton source were found. However, these methods were deemed unsuitable for our purpose due to a poor atom economy, the requirement for difficult to remove protecting groups or challenges incorporating deuterium under standard laboratory conditions. Ultimately, this survey did suggest that Tyr triflate 11a would provide the most direct path to the desired transformation.

While examples of aryl triflate reduction are quite commonly reported in the literature, only three of these reports provided examples of deuterium incorporation. We focused our attention on the work of Sajiki, who described an operationally simple Pd/C catalyzed reduction of aryl triflates using Mg turnings in MeOH at rt. A notable rate acceleration was observed upon addition of a variety of ammonium salts, specifically 1 eq of NH₄OAc. In the course of mechanistic experiments, CH₃OD and CD₄OD were reported to provide regioselective deuterium incorporation, suggesting that the hydroxyl proton was the source of deuterium in the reaction.

Before employing these conditions, we opted to exchange NH₄OAc for NH₄Cl. Although both salts were reported to provide similar reaction rate enhancement, the latter was expected to be less hygroscopic than the former, reducing the chance of undesired hydrogen incorporation later. When 11a was exposed to the reported conditions, we observed by
UPLC-MS 30% conversion to 6a after overnight stirring (Entry 1, Figure 3). The reaction was quickly optimized after observing the effect of 2 eq of Mg was essentially complete after 3 hrs (Entry 2 and 3, Figure 3). Addition of a second bolus of 2 eq of Mg and 1 eq NH₄Cl after 3 hrs resulted in quantitative conversion to 6a after additional 3 hrs at rt (Entry 4, Figure 3) producing the desired product in 92% yield. Concerned the presence of basic Mg(OMe)₂ may lead to racemization, we were delighted to find that 6a displayed the same specific rotation as a commercial standard (−4.7° and −4.4°, respectively) and confirmed by chiral chromatography. In a final modification, 11a was taken forward after brief aqueous work up directly into the reduction reaction leading to isolated 6a in 88% overall yield for both steps from Boc-Tyr-OMe. We were gratified to find that the procedure was well adapted to the incorporation of deuterium. Using crude 11a, substitution of ND₄Cl and CD₃OD into the protocol produced 6b in similar yield with deuterium incorporation of over 90% based on ¹H NMR integration.

With conditions for our key transformation secured, we turned our attention to the fully isotopically labelled synthesis. Key to the success of the scheme would be conditions that did not alter the isotopic distributions already installed in 10. Sandmeyer iodination of 10 proved unexpectedly complex as reported conditions had poor reproducibility with regard to yield or purity. During the optimization efforts we noted that in the time between final addition of nitrite and introduction of iodide, the reaction began to take on a gritty consistency suggesting the diazonium salt was no longer soluble in the aqueous medium. This difficulty was overcome by the use of DMSO as co-solvent, demonstrated in Zhu’s high yielding synthesis of 2,3-trifluoromethyl-4-iodophenol, circumvented these issues giving to 9 in 70% yield reproducibly with high chemical purity after chromatography. Despite the strongly acidic conditions, we were pleased to observe no change in aromatic peak integrations between 10 and 9. Cross coupling of the Negishi reagent of 8 with 9 occurred with a slight modification Jackson’s procedure using 2.5 mol% Pd2(dba) and 5 mol% SPhos. After preparing the Negishi reagent in DMF at 25 °C, catalyst components and 9 were added followed by heating at 40 °C overnight. After aqueous workup and chromatography, 7 was isolated in 85% yield. The specific rotation of 7 was found in line (50.3°) with that of a commercial sample (49.9°) and confirmed by chiral chromatography. Interestingly, when the reaction was carried out using preformed Gen 3 S-Phos pre-catalyst instead of Pd2(dba)₃ and S-Phos, the resulting yield dropped to 38%. We attributed this surprising result to the low basicity of the Negishi reagent, that while compatible with the free hydroxyl present in 9, may therefore be insufficiently basic to deprotonate the pre-catalyst and consequently fail to produce the active catalytic species. The synthesis of 1b was completed after a standard sequence of LiOH·H₂O ester hydrolysis followed by removal of the Boc group with 4M HCl in dioxane to give the HCl salt in 96% yield over two steps. Overall, 1b was obtained from 10 in 57% total yield over four steps.

With the route to prepare 2b in hand, we turned our attention to the preparation of 1b (Scheme 3). As before, triflate 11b was prepared from 7 under standard conditions, subjected to brief aqueous workup and carried forward directly into the reduction step. 10% Pd/C, 2 eq of Mg turnings, and 1 eq of ND₄Cl were introduced and placed under nitrogen at room temperature. After dilution with CD3OD, the reaction stirred 3 hrs at

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room temperature wherein a second bolus of 2 eq of Mg\(^0\) and 1 eq ND4Cl was introduced, followed by a further 3 hrs stirring. After aqueous work with 1 M citric acid and column chromatography, 6c was isolated in 86% yield over both steps. The observed specific rotation of 6c again compared favorably with that of the commercial standard (−4.5° and −4.4°, respectively) demonstrating no loss of optical activity by chiral chromatography. The synthesis was completed as before with ester hydrolysis and Boc deprotection to give 1b in 87% yield over the final two steps. Starting from 10, 1b was obtained in 47% total yield over six steps.

In summary, we have developed a concise, flexible, high yielding synthesis to attain spin-isolated labeled \(^{1}\text{H},^{13}\text{C}\) Tyr 2b and Phe 1a for NMR studies. In developing this route, we were able to overcome several challenges encountered during the preparation and utilization of late stage metabolic precursors 3 and 4 which currently provide the best means of access to spin isolated labelled proteins. Beginning with previously reported aminophenol 10, advanced labelled intermediate 7 is prepared in two steps allowing access to either 1b or 2b in further two or four steps, respectively. Key to the flexibility of the route were conditions allowing for the regioselective deuteration of 7 while maintaining stereochemical purity. Activated as its triflate, we demonstrated 7 was quantitatively reduced by Mg turnings with 10% Pd/C in MeOH accelerated by ammonium salts. These conditions were readily adapted to incorporate deuterium regiospecifically at levels above 90%. Finally, we demonstrated that 1b and 2b can be used to efficiently label Phe and Tyr residues in an expressed protein at concentrations of 15 mg/mL. We feel that the convenient synthesis coupled with high levels of Phe and Tyr residue labelling makes 1b and 2b valuable reagents to enable future application of spin isolated aromatic labelling in protein NMR.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Spin isolated Phenylalanine $1a$ and Tyrosine $2a$, their corresponding pyruvate bioprecursors $3$ and $4$ and the common synthetic intermediate $5$. 
Figure 2.
Aromatic $^1$H,13C-TROSY of 45 kDa recombinant ALK extracellular domain (673–1025) prepared with 50 mg/L culture of precursor 3 and 4 (pyruvate-type) top panel a) and 38 kDa recombinant Src kinase domain (248–531) using 15mg/L of precursor 1 and 2 (amino acid-type) bottom panel a). Up to 7-fold higher Phe incorporation was found vs. Tyr when 3 and 4 were used. In contrast, 1b and 2b gave equal and high incorporation of both Tyr and Phe. Panel b) resonance assignment of Phe and Tyr in highly deuterated proteins up to 50 kDa is obtained by NOESY matching the intra benzylic proton (Hβ) using reagents 1b and 2b in this work.
Figure 3.
Reaction screening to prepare 6a/b from 11a

| Entry | Conditions       | Solvent | Time | Conversion 11:6 |
|-------|------------------|---------|------|-----------------|
| 1     | 1 eq Mg/1 eq NH₄Cl | CH₃OH   | 24   | 70:30 6a        |
| 2     | 2 eq Mg/1 eq NH₄Cl | CH₃OH   | 24   | 50:50 6a        |
| 3     | 2 eq Mg/1 eq NH₄Cl | CH₃OH   | 3    | 50:50 6a        |
| 4     | 2 eq Mg/1 eq NH₄Cl x2 | CH₃OH | 6    | 0:100 6a        |
| 5     | 2 eq Mg/1 eq ND₄Cl x2 | CD₃OD | 6    | 0:100 6b        |
Scheme 1.
Retrosynthetic Design of Spin Isolated Amino Acids 1b and 2b from a single advanced intermediate.
Scheme 2.
Synthesis of Spin Isolated Tyr 2b from Intermediate 10.
Scheme 3.
Preparation of Spin Isolated 1b from Intermediate 7.