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Improved chemical synthesis of *o*-nirtrobenzyl-tyrosine for concise site-specific ^{15}N-tyrosine NMR analysis demonstrated by plant ABA receptor PYL10

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ABSTRACT

The yield of unnatural amino acid *o*-nitrobenzyl-tyrosine (oNBTyr), which was able to recover to natural tyrosine after UV-photocleavage was greatly improved from 20% to 81% by using 2-nitrobenzyl bromide as the nucleophilic reagent. Through genetically introducing ^{15}N-oNBY and consequent photo-cleavage, the site-specific ^{15}N-Tyr NMR analysis of plant ABA (abscisic acid) receptor PYL10 was implemented without any residue variation. This isotope labelling of tyrosine onto protein backbone provides a convenient strategy for NMR analysis.

Nuclear magnetic resonance (NMR) spectroscopy has been proven to be a powerful technique to determine the structure, dynamics, and function of proteins.[1] A variety of NMR pulse sequences have been developed to investigate the local conformation changes of protein during substrate binding or signal transduction processes.[2] However, the crowded NMR spectra of [^{15}N, ^{13}C] fully labelled protein and the difficulties of resonances assignment often represent serious challenges. Simplification of NMR spectra could be achieved by introducing site-specific unnatural amino acid (UAA) isotope labels.[3] Using an orthogonal amber tRNA/tRNA synthetase (tRNA/RS) pair, the desired unnatural amino acid can be incorporated into protein at the amber nonsense codon (TAG) during protein synthesis in *E. coli* cells.[4] Then, the obtained NMR resonance can be assigned to the UAA at the desired location with no ambiguity. Especially, a special class of photocleavable UAAs can transfer to natural amino acids after subjected to light stimulation without disturbing natural protein structure.

o-Nitrobenzyl-*O*-tyrosine (oNBTyr) is a UV-photocleavable unnatural amino acid.[5] After irradiation with 365 nm UV light, oNBTyr loses it photo-caged group and transform back to the natural amino acid tyrosine (Scheme 1). Tyrosine is of great significance in proteins, as it undergoes phosphorylation, sulfation, or hydrogen bond formation, which regulate the protein structure, enzyme activity or interaction between proteins. These advantages make oNBTyr become a unique unnatural amino acid which can introduce an isotope label at specific tyrosine site without causing any protein sequence and structure perturbation. Although oNBTyr was first synthesized by Henry Lester and co-workers in 1998,[6] the yield was very low (only around 20%) which greatly limits its biological applications. In this study, we report an improved oNBTyr synthesis protocol which enabled the synthesis yield of ^{15}N-oNBTyr to be 81%. Then we first incorporated the ^{15}N-oNBTyr into the substrate binding pocket of Arabidopsis thaliana abscisic acid (ABA) receptor PYL10 to investigate its ABA binding process.

The synthesis protocol of oNBTyr is elegant, with only three simple but indispensable steps (Scheme 1). First, the copper salt was used to protect both the amino- and carbonyl-group of tyrosine through chelating reaction in water with basic pH; Then hydroxyl-group of tyrosine was caged with *o*-nitrobenzyl group by substitution reaction using 2-nitrobenzyl chloride and finally Cu^{2+} deprotection was achieved using hydrochloric acid, resulting in the hydrochloride salt of oNBTyr with synthetic yield of around 20%. With a carefully check on oNBTyr synthesis pathway, the nucleophilic substitution reaction (SN_2 in this case) of the second step was considered as the key, which could determine the synthetic yield. As a SN_2 reaction, an important aspect could influence

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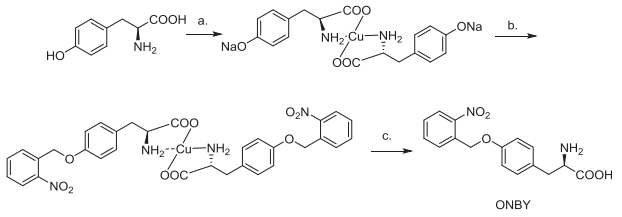
the reactivity is the leaving group that links to the carbon of reactive center. It is known that Br is more active than Cl in SN_2 reactions, especially the Br^- is more capable to take two electrons of its bond to carbon during the reaction than Cl^-.[7]Another evidence is that the bonding energy of C—Br is 280 kJ/mol, lower than C—Cl 397 kJ/mol,[8] makes C—Br bond easier to break in reaction. According to the above description, we assume a simple change of the reactant *o*-nitrobenzyl bromine instead of *o*-nitrobenzyl chloride may improve the synthetic yield, and the result after the modification do meets our assumption. The new total synthesis yield of oNBTyr is 81%, much higher than Henry Lester's report. The ^{15}N labelled tyrosine (Cambridge Isotope Laboratory, MA, U.S.) was applied as the source to synthesize ^{15}N labelled oNBTyr.



Scheme 1. Synthetic route of oNBTyr. Conditions and reagents: (a) $Cu(NO_3)_2{\cdot}3H_2O$, NaOH, H_2O, 60 °C, 30 min; (b) 2-NO_2BnBr, NaOH, H_2O/MeOH/DMF, rt, 24 h; (c) 1.0 M HCl, rt, 1 h.

After a series of oNBTyr decaging assays and ^1H NMR analysis (supporting information), the UV irradiation condition was optimized (365 nm UV light at 3.5 mW/cm^2, 10 min) to cleave the *o*-nitrobenzyl group from oNBTyr, resulting in native amino acid tyrosine.

After obtaining the ^{15}N-labelled oNBTyr, efficiency of site-specific oNBTyr labelling system was tested using model protein GB1 (Fig. 1), a *Streptococcus* protein GB1 domain consisting 57 amino acids, used for methodology studies.[9] The coding sequence of GB1 was cloned into pET21b plasmid, resulting in a C-terminal 6× His-tag. Site-directed mutagenesis was used to introduce the Y33TAG mutation into the GB1 gene. The pSupC plasmid was prepared, containing amber tRNA and aminoacyl-tRNA synthetase selected for oNBTyr. These two plasmids were co-transformed into *E. coli* BL21(DE3)-Gold cells for further protein expression (Fig. 1a). Cells were grown at 37 °C in LB medium to an OD_{600} of 0.8, then ^{15}N-oNBTyr and isopropyl b-D-thiogalactopyranoside (IPTG) were added to a final concentration of 1 mM and 0.2 mM, respectively, into the culture to induce the expression of GB1-Y33oNBTyr. The His-tagged GB1 were purified using Ni^{2+}-NTA affinity column (protein sequence and ESI-MS spectrum see Supporting information). SDS-PAGE confirmed the high purity of the GB1 sample (Fig. 1b). A 800 mL scale expression with above condition gave a protein yield of 17 mg/L.

With the ^{15}N-labelled oNBTyr incorporated at Tyr33 site of GB1 protein, two dimensional ^{15}N HSQC spectra were acquired to analyze the photo-decaging of the oNBTyr before or after 10 min 365 nm UV irradiation (Fig. 1c). Two peaks were observed for the sample before UV irradiation, representing the caging or decaging conformations of the Tyr33 residue. ^1H chemical shift of these two conformations were 7.95 ppm and 8.24 ppm respectively. The decaging conformation of the sample was probably due to light stimulated photo-cleavage reaction during the protein purification. After UV irradiation, only the peak at 8.24 ppm was left, strongly indicating that the oNBTyr at Tyr33 was completely converted to native tyrosine, resulting in site-specific ^{15}N labelled tyrosine in GB1 without any structural perturbations.

Abscisic acid (ABA) is one of the most important phyto-hormones, and playing essential roles in plant growth, development,

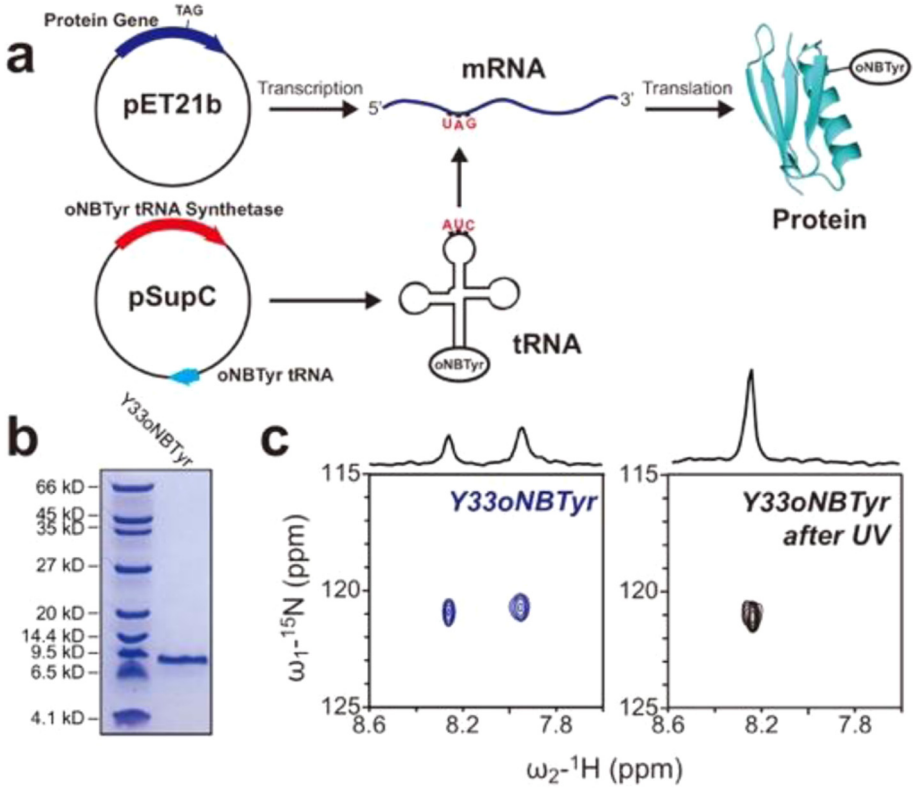


Fig. 1. (a) The experimental principle applied to synthesize site-specific oNBTyr labelled proteins. (b) Coomassie-stained SDS-PAGE gel of the purified GB1-Y33oNBTyr protein. The molecular weight of GB1 was about 7 kDa. (c) 1H–15N HSQC spectra of GB1 mutants with ^{15}N-labelled oNBTyr incorporated at Y33 before (blue) and after (black) UV-cleavage.