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Facile synthesis and application of uniformly ^{13}C, ^{15}N-labeled phosphotyrosine for ligand binding studies

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Abstract—The first synthesis of [U-^{13}C, ^{15}N] labeled phosphotyrosine is described. Preliminary studies toward the binding of phosphotyrosine to an SH2 domain have been performed by means of heteronuclear NMR.

Protein phosphorylation is one of the primary means of generating both transmembrane and intracellular signals to coordinate a vast array of metabolic processes. Rapid phosphorylation–dephosphorylation by protein kinases and phosphatases accounts for the on and off signals that typically result in a signal amplification cascade. In eukaryotes, phosphorylation occurs primarily on the amino acid residues serine, threonine, and tyrosine. Of these, tyrosine in particular plays a major role in signal transduction.[1] Phosphorylated tyrosine residues serve as binding sites for other signaling proteins containing phosphotyrosine recognition elements such as the Src homology 2 (SH2),[2] phosphotyrosine binding (PTB),[3] and certain Forkhead-associated (FHA)[4] domains. Malfunctioning of this molecular machinery can lead to a whole range of diseases including several types of cancer.[5]

Selective inhibition of different components of a signal transduction process is of great importance in the elucidation of the mechanism and its biological consequences and may well be a first step in the design of therapeutically active compounds. SH2 domains in particular are considered to be promising therapeutic targets,[6] but the development of high specificity small molecule inhibitors has met with moderate success.[7] More detailed understanding of the ligand–protein interaction could help to overcome these difficulties.

One approach to investigate the structure and behavior of the phosphotyrosine ligand in the signaling cascade is the synthesis of phosphotyrosine mimetics. Over the years, several types of mimetics have appeared in the literature, all of which contain a functional group resembling the properties of the natural phosphate.[8] These include (α-substituted) phosphonates,[9] malonylated phenylalanines or tyrosines,[10] and monocarboxylated aromatic amino acids.[11] Although several of these mimetics bind to SH2 domains, they all show significantly lower affinity than the natural ligand. In order to rationalize this reduced affinity it is important to first understand the interaction of phosphotyrosine itself with a receptor, such as the SH2 domain, in detail. In this framework, the ready availability of stable isotope labeled phosphotyrosine would allow a detailed study of binding through focusing exclusively on the relatively simple NMR spectrum of the ligand providing access to hitherto unavailable information.

Herein we describe the first and efficient synthesis of [U-^{13}C, ^{15}N] labeled phosphotyrosine and the initial results of an NMR-study aimed at better understanding the interaction of this compound with an SH2 domain.

Commercially available Fmoc-[U-^{13}C, ^{15}N]-Tyr(O-*t*-Bu)-OH[12] was allylated under exclusion of light using silver carbonate and allyl bromide in DMF to afford crude allyl ester **2**. Acidolysis of the *tert*-butyl group in crude **2** with TFA afforded phenol **3** which was phosphorylated in a two-step procedure without purification of the intermediates. Thus, phenol **3** was phosphitylated with (*N*,*N*-diisopropylamino)-dibenzylphosphite under

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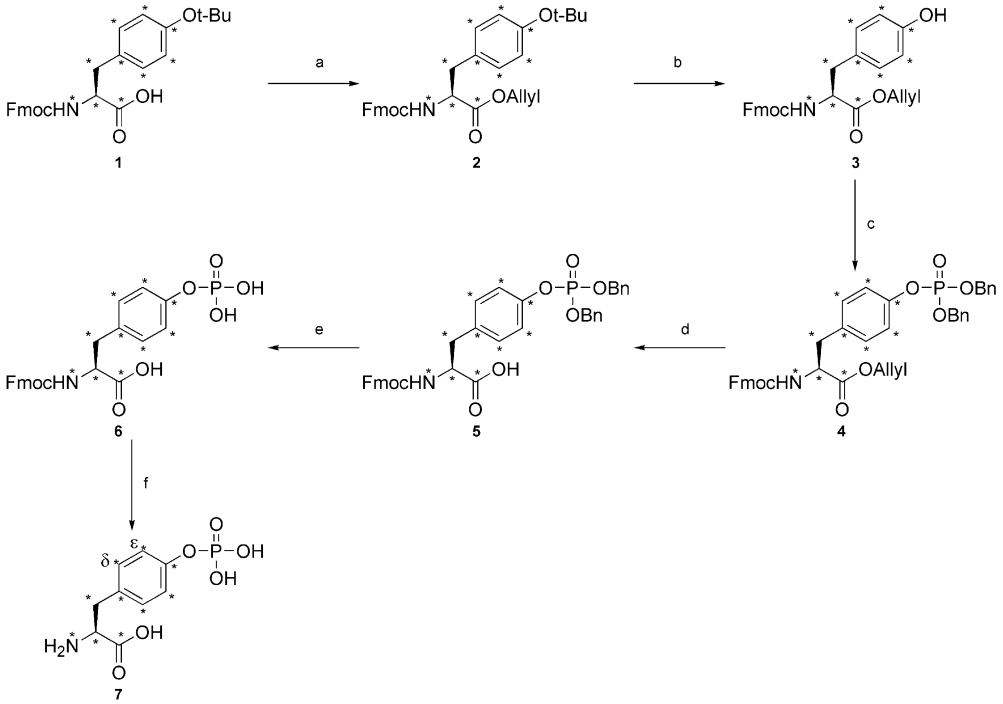
the influence of benzimidazolium triflate[13] and the intermediate phosphite triester was oxidized with *tert*-butylhydroperoxide to give **4** (Scheme 1).

Treatment of crude **4** with acetic acid, tributyltin hydride, and palladium (II), and purification by column chromatography afforded **5** in 62% over four steps. Debenzylation of **5** using a mixture of TFA/H_2O/TIS 95:2.5:2.5 and removal of the Fmoc group yielded, after HPLC purification, fully unprotected [U-^{13}C,^{15}N]-phosphotyrosine **7** in 40% yield over two steps. The structure of **7** was confirmed by LC–MS, HRMS, and NMR analysis.

Previous studies have demonstrated chemical shift perturbations to the C-terminal SH2 domain of the β subunit of the enzyme phosphoinositol 3 kinase (CSH2) upon addition of phosphotyrosine-containing peptides.[14] In these studies addition of substoichiometric amounts of peptide to the CSH2 domain resulted in two sets of resonances indicating the peptide was in slow exchange on the NMR time scale. Titration of phosphotyrosine into ^{15}N labeled CSH2 domain resulted in changes in the chemical shift of protein nuclei which were gradual and dependent on the amount of compound added.[15] This observation indicates that compound **7** is in rapid exchange with the protein on the NMR time scale (i.e., $\Delta\delta$ (Hz) << k_{off}). However, the chemical shift assignment of phosphotyrosine, particularly in the bound state, is not available. The availability of both ^{1}H and ^{13}C chemical shifts of phosphotyrosine would enable validation of, for example, ab initio studies of ligand–protein interaction.

To address this issue a titration experiment was conducted in which a constant amount of CSH2 was treated with a variable amount of [U-^{13}C, ^{15}N] phosphotyrosine as ligand. We performed a [^{13}C,^{1}H] HSQC experiment to acquire both ^{1}H and ^{13}C resonances of phosphotyrosine in solution. Focusing on the aromatic moiety, the chemical shifts of **7** in the absence of protein are Cδ 131 ppm, Hδ 7.17 ppm, Cε 121 ppm, and Hε 7.12 ppm (Fig. 1). Not surprisingly, the chemical shifts at the ε position of both the ^{13}C and ^{1}H nuclei are considerably downfield shifted with respect to tyrosine, while at the δ position they remain relatively unchanged. Upon addition of protein, the chemical shift of both Cε and Hε shows concentration-dependent changes indicating that **7** is in rapid exchange with the protein. The chemical shift of Hε is about 7.80 ppm in the fully bound state, while that of Cε is about 121.4 ppm. Note that the peak corresponding to the highest protein–ligand ratio (cyan in Fig. 1) is missing. This can be explained by a poor signal to noise ratio due to the relatively low concentration of **7** in combination with broadened linewidth caused by averaging between the bound and free states. In contrast, the Cδ–Hδ correlation exhibits very different behavior. Instead of a gradually shifting position upon addition of protein, two distinct peaks appear at intermediate ratios of ligand to protein (Fig. 1). As the ratio of protein to the ligand **7** increases, the peak corresponding to free **7** decreases and the new peak, corresponding to bound **7**, increases. The peak corresponding to the bound state has coordinates of 7.12 ppm for Hδ and 131.1 ppm for Cδ. This behavior is indicative of slow exchange on the NMR timescale. In light of the rapid exchange observed for the Hε/Cε



Scheme 1. Reagents and conditions: (a) $AgCO_3$, allyl bromide, DMF; (b) TFA/H_2O, 95:5; (c) (*N*,*N*-diisopropylamino)-dibenzyl phosphite, benzimidazolium triflate, ACN then *tert*-butylhydroperoxide; (d) AcOH, tributyltin hydride, $Pd(PPh_3)Cl_2$, 50% THF/DCM, 62% over four steps; (e) TFA/TIS/H_2O 95:2.5:2.5, 2× 3 h; (f) 20% piperidine/DMF, HPLC, 40% over two steps.

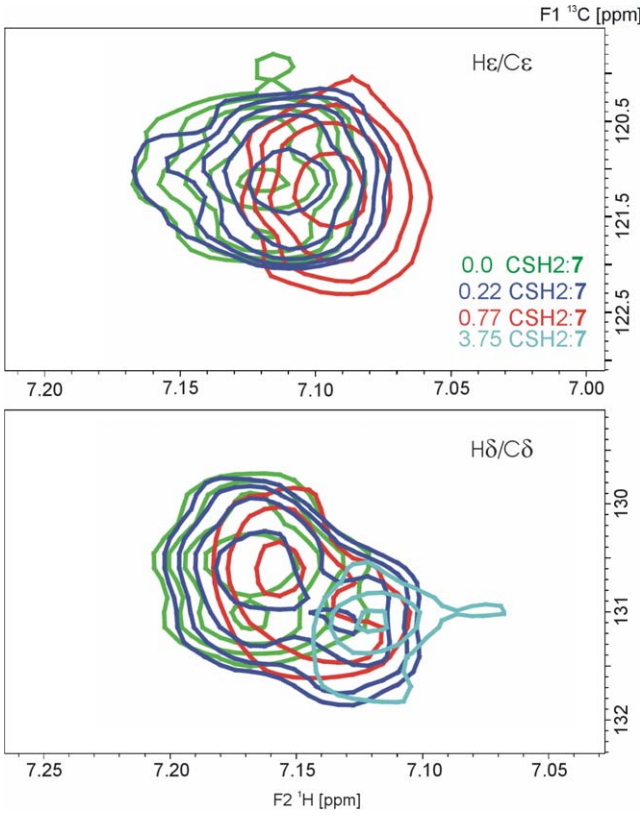


Figure 1. Titration of [U-^{13}C, ^{15}N]-phosphotyrosine (**7**) with the CSH2 domain. The upper panel shows the Hε–Cε correlation while the lower panel shows the Hδ–Cδ correlation of 7. The ratio of CSH2:7 used for each experiment is indicated. At the lowest concentration, **7** was present at 33 μM. An HSQC pulse sequence was used with 96 increments of 512 transients per increment. The ^{13}C offset was placed in the middle of the aromatic region at 120 ppm. The $^{1}J_{HC}$ was assumed to be 160 Hz for the aromatic correlations. All data were recorded at 14.1 T (600 MHz ^{1}H frequency). Data in both dimensions were apodized with a sine^{1} function prior to Fourier transformation.

correlation, which corresponds well with the relatively weak binding (K_D = 0.6 mM)[15] observed for **7** and the CSH2 domain, this slow exchange is unexpected and, at present, unexplained.

In conclusion, we have shown that [U-^{13}C, ^{15}N]-phosphotyrosine (**7**) can be made via an efficient six-step sequence in a 23% overall yield after HPLC purification. Protected intermediate **5** can also be used for solid phase peptide synthesis according to established procedures.[16] The NMR analysis of labeled phosphotyrosine ligand in the presence and absence of a protein has provided the aromatic carbon resonance assignment in the bound state. An unexpected observation is the apparent difference in kinetic behavior of the Hε/Cε and Hδ/Cδ correlations. Whether or not this difference is related to the specificity for phosphotyrosine over mimetics is not yet known and will be the subject of future investigation.

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