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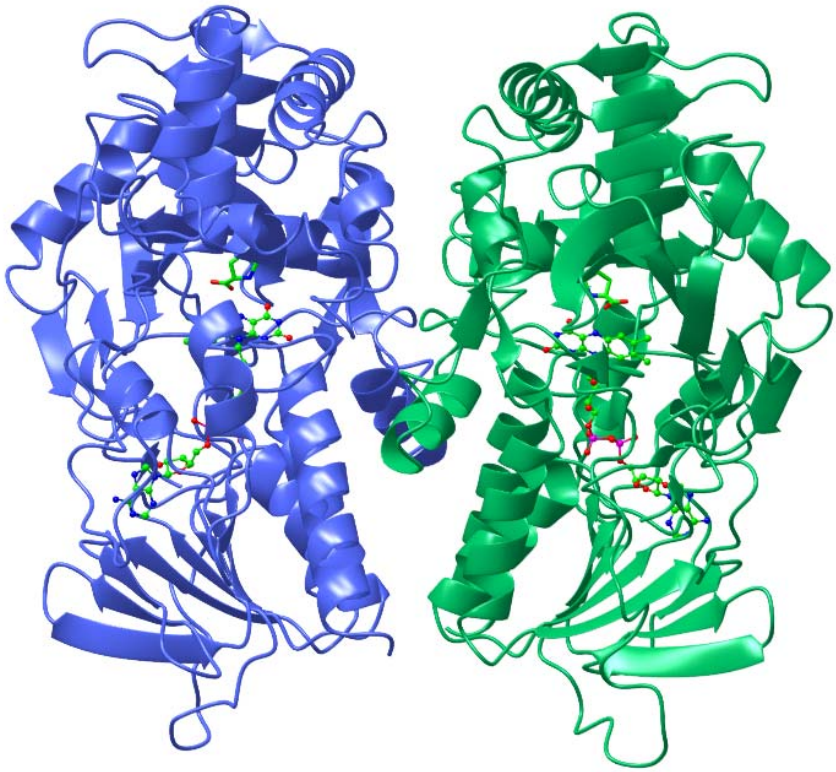

# An unnatural amino acid that mimics phosphotyrosine†‡

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**By replacing the phosphate group in *p*Tyr with an isoxazole carboxylic acid, a novel unnatural amino acid has been successfully synthesized. Subsequently, its incorporation into a known PPI (protein–protein interaction) inhibitor of STAT3 protein (ISS 610) generated I2 which showed reasonable anti-STAT3 activity in both fluorescence polarization and cell-proliferation experiments.**

Protein–protein interaction (PPI) mediates a large number of important regulatory pathways in cellular processes.[1] Inappropriate interactions are known to cause many human diseases including cancer and obesity. Small molecule inhibitors capable of disrupting protein–protein interaction therefore offer unique opportunities in drug discovery, especially against less common therapeutic targets such as transcription factors.[2] PPI inhibitors of STAT3 are such examples.[3] The signal transducer and activator of transcription (STAT) family of proteins, including STAT3, are mediators of cytokine and growth factor responses, and control cellular growth and differentiation, survival, development and inflammation.[4] They are members of the Src homology 2 (SH2) domain-containing proteins, which also include many other adaptor proteins, phosphatases (*i.e.* SHP1 and SHP2) and kinases (*i.e.* Src and Abl).[5] SH2 domains are phosphotyrosine (*p*Tyr)-binding protein domains made of ~100 amino acids.[4,6] In recent years, they have become attractive targets for developing new PPI inhibitors and potential therapeutic drugs.[7] In the case of STAT3 inhibitors, it is known that the activation of STAT3 is initiated by the phosphorylation of a key tyrosine residue near its C-terminus, which triggers protein dimerization *via* SH2–*p*Tyr interaction, resulting in subsequent nuclear translocation, binding to specific DNA-response elements and inducing gene transcription.[8] Although there are several proteins in the STAT family, so far only STAT3 is shown to be expressed constitutively in a variety of cancer cells, making it a good target for cancer therapy and the corresponding PPI inhibitors potential antitumor agents.[3]

Most SH2 domain-targeting inhibitors are *p*Tyr containing peptides or peptidomimetics, in which the phosphotyrosyl group is the most critical element for molecular recognition/tight binding to the SH2 domain. However, its hydrolytic instability (mainly due to enzymatic hydrolysis by cellular protein tyrosine phosphatases or PTPs) and predominantly anionic nature at physiologic pH practically render the corresponding *p*Tyr containing inhibitors to possess poor cell permeability and bioavailability. Numerous *p*Tyr bioisosteres have been developed (Fig. 1a),[7c,d,9] most of which, however, are either still highly charged (**2a–c**), or poor mimetics (**3–5**; therefore possess low SH2-binding affinity). Herein, we report the design and chemical synthesis of a novel unnatural amino acid, **6** (Fig. 1a), in which the phosphate group in *p*Tyr was replaced with a non-hydrolyzable isoxazole carboxylic acid. The isoxazole carboxylic acid was previously shown to be a good cell-permeable bioisostere of the phosphate, and has been incorporated into small molecule inhibitors of PTPs.[10] Our finding here represents the first example in which this moiety has been successfully introduced as the side-chain of an amino acid. We further show this unnatural amino acid can be readily incorporated into SH2 domain inhibitors, *i.e.* **ISS 610** (a previously reported STAT3 PPI inhibitor[3a,c]), to give a new inhibitor **I2** which possesses more desirable pharmacological properties (cell permeability and biostability) but still retains most of its inhibitory activity against STAT3 (Fig. 1b).

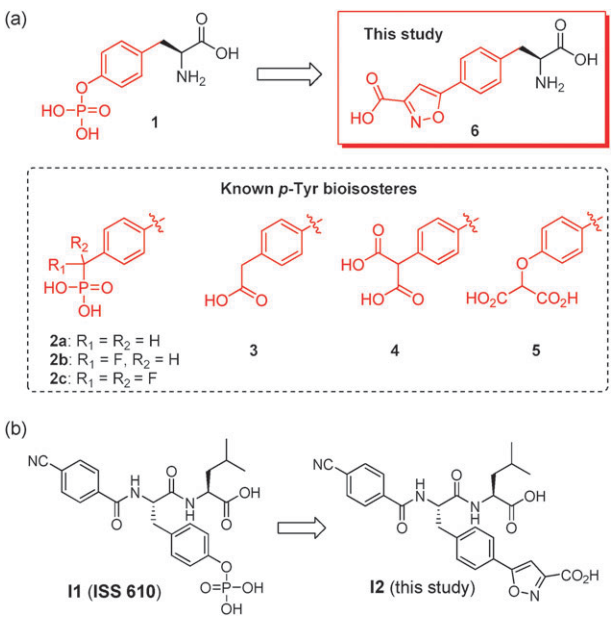


**Fig. 1** (a) Structures of *p*Tyr (**1**), representative examples of previously reported *p*Tyr bioisosteres (**2–5**), and the one developed from this study (**6**). (b) Structures of **ISS 610** (a previously reported STAT3 inhibitor[3a,c]) and **I2** (an improved version with incorporation of **6**).

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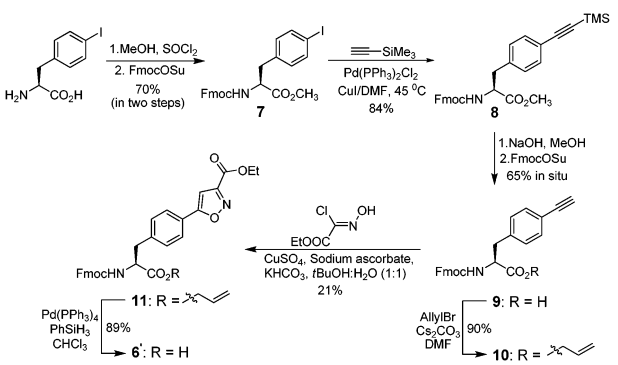
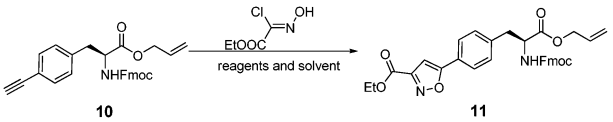
The Fmoc-protected form of the unnatural amino acid, **6′**, was synthesized in several steps from the commercially available 4-iodophenylalanine, as shown in Scheme 1. Fmoc was chosen as the protecting group for the α-amino group in **6′** so that it's compatible with standard Fmoc-based solid-phase peptide synthesis. The isoxazole carboxylic acid side-chain was protected in the form of an ethyl ester as it is orthogonal to Fmoc chemistry and could be deprotected by LiOH.[10b,c] Briefly, the Fmoc-protected methyl ester of 4-iodophenylalanine, **7**, was subjected to Sonogashira coupling conditions with $Pd(PPh_3)_2Cl_2$–CuI in DMF, in the presence of TMS-protected acetylene, to give **8** in 84% yield. All three protecting groups were then removed by NaOH treatment, followed by Fmoc protection of the *N*-α-amino group to give **9** (65% yield in two steps). Subsequent protection of **9** in the form of an allyl ester gave **10** (90% yield). Next, the key isoxazole ring in **11** was formed using a recently published procedure,[11] under the Cu(I)-catalyzed 1,3-dipolar cycloaddition conditions between the terminal alkyne in **10** and ethyl chlorooximidoacetate. Previously, it was found electron-deficient nitrile oxides in general react sluggishly.[11a] Attempts were therefore made to optimize the reaction conditions (Table 1); the best yield obtained was with $CuSO_4$–sodium ascorbate in $KHCO_3$ with (1 : 1) *t*BuOH–$H_2O$ cosolvent, giving **11** in 21% and 23% yield in 1 d and 5 d, respectively. Finally, deprotection of the allyl group by $Pd(PPh_3)_4$–$PhSiH_3$ afforded the Fmoc-protected unnatural amino acid precursor **6′**. No sign of racemization was observed throughout the whole synthesis and in the final unnatural amino acid **6′**.

Next, the two STAT3 PPI inhibitors, **I1** and **I2** (Fig. 1), were synthesized. **I1**, or **ISS 610**, is a peptidomimetic inhibitor derived from the STAT3 SH2 domain-binding *p*Tyr (Y*) peptide PY*LKTK.[3a] **ISS 610** inhibits STAT3:STAT3 dimer ($IC_{50}$ = 42 μM from a DNA-binding assay[3a]) and was shown to induce cell growth inhibition and apoptosis of human cancer cells that harbor constitutively active STAT3, including MDA-MB-435 and MDA-MB-231 (human breast), NIH3T3 (fibroblast) and T47D (human breast) cells.[3c] **ISS 610**, however, has limited cell permeability and hydrolytic stability due to its *p*Tyr group. We reasoned that replacing *p*Tyr in **ISS 610** with our newly developed unnatural amino acid **6** (giving **I2**) should improve both aspects, making **I2** a potentially better STAT3 inhibitor for therapeutic intervention. Modeling experiments were carried out by docking both **I1** and **I2** to the SH2 domain of STAT3; results indicated that both




**Scheme 1** Synthesis of **6′** – the Fmoc-protected form of **6**.

**Table 1** Optimization of isoxazole ring formation under Cu(I)-catalyzed, 1,3-dipolar cycloaddition conditions



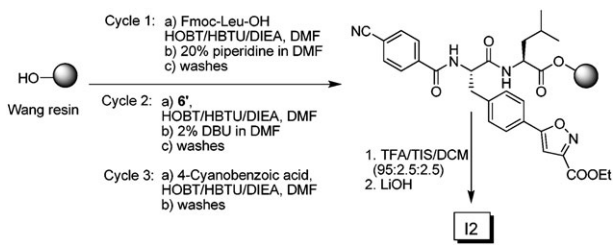

10 11

| Entry | Reagents (A or B)[a] | Solvent | Time/day | Yield (%) |
|---|---|---|---|---|
| 1 | A | *t*BuOH–$H_2O$ (1 : 1) | 1 | 21 |
| 2 | A | *t*BuOH–$H_2O$ (1 : 1) | 5 | 23 |
| 3 | A | DCM–$H_2O$ (1 : 1) | 1 | 20 |
| 4 | A | THF–$H_2O$ (1 : 1) | 1 | 19 |
| 5 | B | THF | 1 | 15 |

[a] A: $CuSO_4$–sodium ascorbate–$KHCO_3$ (0.05/0.1/4 equiv.); B: triethylamine (2 equiv.).

inhibitors bind to the expected site in the protein with similar binding modes (Fig. S6 in the ESI‡). Both inhibitors were subsequently synthesized using Fmoc-based solid-phase peptide synthesis protocols (see Scheme 2 for synthesis of **I2**). Upon resin cleavage, the ethyl group was removed by LiOH treatment, delivering the final inhibitor **I2** which was further purified by preparative HPLC to homogeneity (as judged by $^1$H NMR) before biological screenings.

**I1** and **I2** were subsequently tested for their *in vitro* binding to the STAT3 SH2 domain in a fluorescence polarization (FP) experiment, and inhibition activity against the T47D cancer cell line in a cell proliferation assay (Fig. 2). The FP experiment contained the recombinantly expressed SH2 domain of STAT3, a fluorescently labeled SH2-binding peptide (Fluorescein-GY*LPQTV), and different concentrations of each inhibitor. As shown in Fig. 2a, **I2** ($IC_{50}$ = 144 ± 36 μM) was able to retain most of the binding affinity of **I1** ($IC_{50}$ = 83 ± 21 μM) to STAT3,[12] indicating our unnatural amino acid indeed serves as a good mimic of *p*Tyr. In the cell proliferation assay (Fig. 2b), we observed a corresponding dose-dependent inhibition of **I2** towards T47D cells. More importantly under the same concentration (250 μM), **I2** was at least as effective as **I1** (see bars labeled with * in Fig. 2b), suggesting that although **I2** may have weaker binding towards the SH2 domain of STAT3 *in vitro* than **I1**, its better cell permeability and hydrolytic stability have apparently led to an improved anti-tumor activity in our cell-based assays (see Fig. S7 and Table S1 in ESI‡). The specificity of **I2** was further tested against PTP1B (a PTP previously known to be inhibited by isoxazole-containing compounds[10]); little or no inhibition of PTP1B by up to 2 mM of **I2** was observed, indicating **I2** is likely a specific inhibitor of the STAT3 SH2 domain. Although **I2** (similar to **I1**) is a rather poor inhibitor, future improvement may be made by




**Scheme 2** Solid-phase synthesis of **I2**.

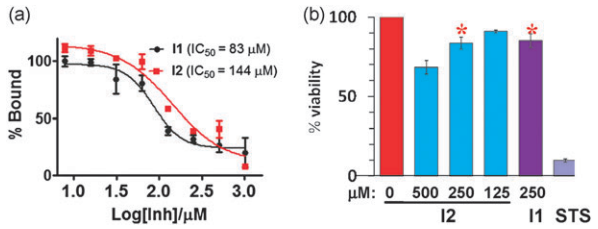



**Fig. 2** (a) $IC_{50}$ of the two inhibitors determined by fluorescence polarization (FP) experiments. (b) Relative % of T47D cell viability in the presence of the two inhibitors, **I2** (500 μM, 250 μM and 125 μM) and **I1** (250 μM). Negative control: DMSO only (0.56% final conc.). Positive control: staurosporine (STS, 500 nM). ± SD was based on average of two independent experiments.

modifications of other portions in **I1**, either rationally or combinatorially.[3]

In summary, we have synthesized a novel unnatural amino acid which mimics phosphotyrosine but possesses better cell permeability and hydrolytic stability. Its incorporation into a known STAT3 SH2 domain inhibitor has confirmed our design principle. Future work will focus on incorporation of this amino acid into other *p*Tyr-containing biologically active compounds. This new *p*Tyr mimic, together with other newly developed approaches,[13] will provide expanded chemical tools for future studies of PTP biology.

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