Letter

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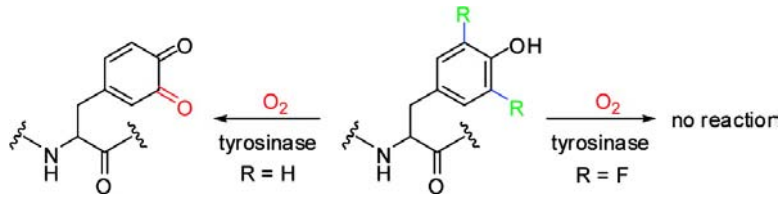

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Synthesis of 3,5-Difluorotyrosine-Containing Peptides: Application in Substrate Profiling of Protein Tyrosine Phosphatases

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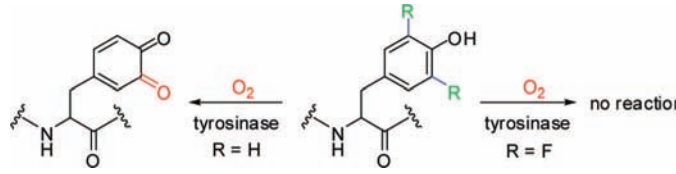
ABSTRACT



Fully protected 3,5-difluorotyrosine (F_2Y), Fmoc-F_2Y(tBu)-OH, is efficiently prepared by a chemoenzymatic process and incorporated into individual peptides and combinatorial peptide libraries. The F_2Y-containing peptides display kinetic properties toward protein tyrosine phosphatases (PTPs) similar to their corresponding tyrosine-containing counterparts but are resistant to tyrosinase action. These properties make F_2Y a useful tyrosine surrogate during peptide library screening for optimal PTP substrates.

Replacement of a hydrogen with fluorine results in a small increase in the molecular size but often dramatically different physical, chemical, and biological properties of the molecule.[1] As such, fluorinated compounds provide useful mechanistic probes of enzyme-catalyzed reactions and other biological processes. For example, ring fluorinated analogues of tyrosine have been used to examine the catalytic mechanisms of tyrosinase,[2] tyrosine phenol-lyase,[3] protein tyrosine kinase and phosphatase,[4,5] Δ5−3-ketosteroid isomerase,[6] and ribonucleotide reductase.[7]

Our own interest in fluorotyrosines stems from our ongoing studies on protein tyrosine phosphatases (PTPs), a large family of enzymes that catalyze the hydrolysis of phosphotyrosine (pY) in proteins back to tyrosine and inorganic phosphate. A major challenge in the PTP field is to determine the physiological substrates and cellular function of these enzymes. We recently developed a combinatorial library method to profile the substrate specificity of PTPs and subsequently used the specificity information to predict the protein substrates of the PTPs.[8] A key element of the technique involved selective derivatization of the reaction product (i.e., tyrosine) with a chemical tag (e.g., biotin). This was accomplished by first oxidizing the tyrosine side chain into an orthoquinone with O_2 and tyrosinase, followed by conjugate addition with biotin-hydrazide. To prevent false positives from unreacted substrates, tyrosine was excluded from the library. However, PTPs may require tyrosine residues for optimal binding and catalysis. Thus, we envisaged the inclusion of a fluorinated tyrosine into the peptide

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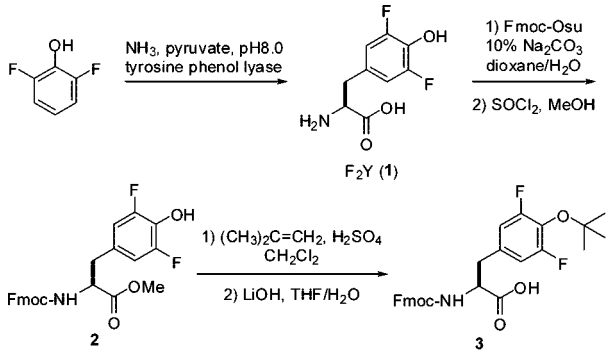
library as a tyrosinase-resistant tyrosine surrogate. Among all of the known mono-, di-, and multiply fluorinated tyrosine analogues, we reasoned that 3,5-difluorotyrosine (F_2Y, compound **1** in Scheme 1) should contain the minimal

Scheme 1. Synthesis of Fully Protected F_2Y



number of fluorine substitutions to render it resistant to tyrosinase action. Because F and H atoms have similar van der Waals radii (1.35 Å for F and 1.10 Å for H), Tyr and F_2Y are essentially isosteric, although the side chain of F_2Y has a lower pK_a value (7.2) than that of Tyr (9.9).[9]

F_2Y has previously been synthesized both chemically[10] and enzymatically.[3,4,7] Synthesis of F_2Y-containing peptides employed side chain unprotected Fmoc-F_2Y-OH, and the resulting peptides were purified by HPLC.[4,7] The reported peptides were either very short or contained F_2Y near their N-termini (therefore no repeated coupling reactions after incorporation of F_2Y), and each contained only a single F_2Y residue. We felt that the unprotected F_2Y side chain might be problematic with peptide library synthesis, during which more forcing coupling conditions are typically employed to drive reactions to completion, some library members will contain multiple F_2Y residues, and HPLC purification is not an option. In this report, we describe the synthesis of fully protected F_2Y, its incorporation into peptides and peptide libraries, and its activity against PTPs in comparison with the tyrosine counterparts.

We employed the enzymatic systhesis originally developed by Phillips and co-workers[3] to prepare multigram quantities of F_2Y from 2,6-difluorophenol, pyruvate, and NH_3 (Scheme 1). Treatment of F_2Y with *N*-(9-fluorenylmethylcarbonyloxy)succinimide in 10% sodium carbonate gave N$^{\alpha}$-Fmoc-F_2Y-OH, which was subsequently converted into its methyl ester **2** using thionyl chloride in refluxing methanol. The phenol group was next protected as a *tert*-butyl ether by treatment with isobutylene and H_2SO_4. Finally, hydrolysis of the methyl ester by LiOH in THF/H_2O gave the desired N$^{\alpha}$-Fmoc-F_2Y(tBu)-OH (**3**) in 24% overall yield (from F_2Y).

Three F_2Y-containing peptides and their corresponding Tyr-containing counterparts were synthesized on the solid phase by using standard Fmoc/HBTU chemistry (Table 1,

Table 1. Comparison of the Kinetic Constants of F_2Y- and Tyr-Containing Peptides against PTP1B (pH 7.4)

peptide	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (μM^{-1} s^{-1})
DNLF$_{2Y}$pYWD-NH$_2$ (**4**)	36 ± 1	3.6 ± 0.4	10.0
DNLYpYWD-NH$_2$ (**5**)	32 ± 1	5.8 ± 0.8	5.5
DDTF$_{2Y}$DpYAA-NH$_2$ (**6**)	36 ± 2	13 ± 3	2.7
DDTYDpYAA-NH$_2$ (**7**)	33 ± 1	14 ± 2	2.3
REF$_{2Y}$EFpYAA-NH$_2$ (**8**)	44 ± 2	14 ± 2	3.2
REYEFpYAA-NH$_2$ (**9**)	33 ± 1	6.0 ± 0.7	5.4

compounds **4**−**9**). Peptide **5** is derived from a known pY motif of receptor protein tyrosine kinase erbB2,[11] whereas peptides **7** and **9** are analogous to the prefered substrates of PTP1B (the prototypical PTP), previously identified from a peptide library.[8] These peptides contain F_2Y (or Tyr) at the pY-3, pY-2, or pY-1 position (relative to pY, which is designated as position 0). Earlier studies have shown that residues at the N-terminal side of pY are most critical in defining the substrate specificity of PTPs.[12] During peptide synthesis, N$^{\alpha}$-Fmoc-F_2Y(tBu)-OH was efficiently incorporated into peptides without incidence (as judged by ninhydrin tests), despite that it was used at only 1.5 equiv (4 equiv was used for all other amino acids). Reversed-phase HPLC analysis of the crude peptides showed that in each case the desired peptide was the major product (see Figure 1, for

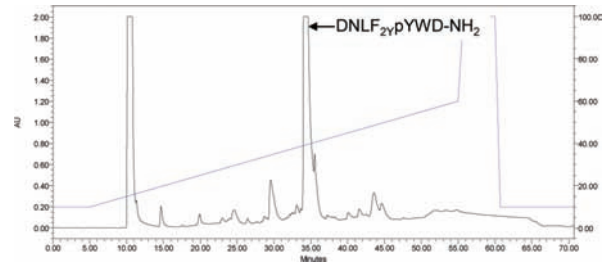


Figure 1. HPLC analysis of peptide **4** on a C-18 column, eluted with a linear gradient of 10−60% CH_3CN in H_2O containing 0.1% trifluoroacetic acid over 50 min.

example). Peptides **4**−**9** were purified by preparative HPLC, and their kinetic activities toward PTP1B (i.e., k_{cat}, K_M, and k_{cat}/K_M values) were determined at pH 7.4 (Table 1). The data show that substitution of F_2Y for tyrosine resulted in minimal changes in kinetic constants (≤2-fold, which is within the margin of experimental error). Thus, F_2Y is a good functional mimic of tyrosine, in terms of binding to the active site of PTPs.

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Next, a pair of Tyr- and F_2Y-containing peptides (peptides **8** and **9** in Table 1) were tested for activity against tyrosinase. The peptides were individually treated with tyrosinase in the presence of atmospheric O_2 and excess biotin hydrazide. Reversed-phase HPLC/MS analysis of the reaction mixtures revealed that the Tyr-containing peptide REYEFpYAA was quantitatively converted into a new species, which had an increased retention time (from 32.0 to 34.5 min) and molecular mass (from *m*/*z* 1168.4. to 1440.7) (Figure S1 in Supporting Information). The increase of 272.3 amu in molecular mass is consistent with the addition of a single biotin hydrazide molecule to the tyrosine side chain. As expected, the F_2Y-containing peptide (**8**) was completely resistant to tyrosinase action.

Finally, to test whether F_2Y is compatible with peptide library synthesis, screening, and postscreening sequence identification, we designed a pY peptide library containing five random positions immediately N-terminal to pY [SAXXXXXpYAABBRM-resin, where B is β-alanine and X is F_2Y, norleucine (Nle), or any of the 17 proteinogenic amino acids excluding Met, Cys, and Tyr]. The library was synthesized on polyethylene glycol polyacrylamide (PEGA) resin[13] by the split-and-pool method.[14] A portion of the resulting one-bead-one-compound (OBOC) library (~40 000 beads) was treated with 1.0 nM PTP1B for 20 min (at pH 7.4), followed by incubation with 1.2 μM mushroom tyrosinase in the presence of atmospheric O_2 and 6 mM 3-methyl-2-benzothiazolinonehydrazone (MBTH). Under these conditions, only beads that carried the most preferred substrates of PTP1B were dephosphorylated (usually only partially). The exposed tyrosine side chain was subsequently oxidized by the excess tyrosinase activity into an ortho-quinone, which was trapped by reaction with MBTH to form a dark pink pigment (Figure 2a).[15] Thus, as a result of this reaction cascade, a bead carrying a preferred PTP1B substrate would turn pink/red, whereas beads carrying poor substrates would not. This was indeed the case (Figure 2b). The pink/red colored beads were removed from the library and individually sequenced by partial Edman degradation/mass spectrometry[16] to reveal the most preferred PTP1B substrates (Table 2). Figure 2c shows an example of the MS spectrum derived from one of the pink beads (bead No. 2 in Table 2), carrying the sequence of SAVITDF$_{2Y}$pYAABBRM. Our results are in agreement with earlier reports that PTP1B prefers acidic residues at the N-terminal side of pY, especially at the pY-2 position.[8,12,17] A control

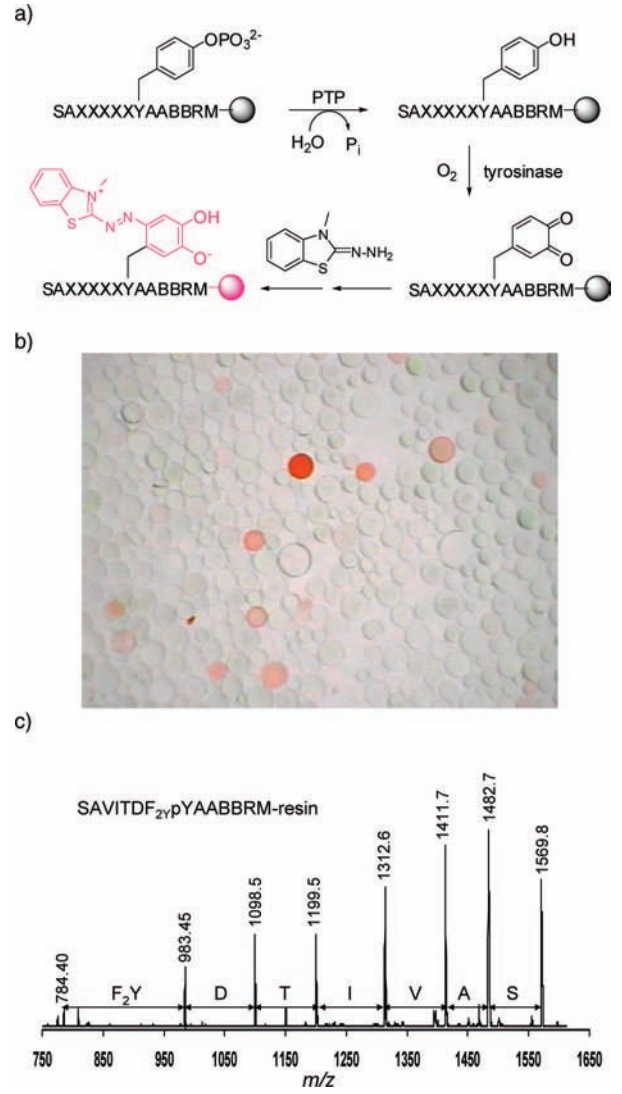

Figure 2. (a) Reactions involved in library screening against PTP and bead coloration. (b) A portion of the OBOC pY library after treatment with PTP1B (1.0 nM) and tyrosinase (1.2 μM) (viewed under a disecting microscope). (c) MALDI-TOF spectrum of peptide SAVITDF$_{2Y}$pYAABBRM* and its truncation products after partial Edman degradation (derived from a single PEGA resin bead). M*, homoserine lactone.

Table 2. Partial List of the Selected PTP1B Substrates[a]

bead no.	peptide sequence
1	QDVDApYAA
2	VITDF$_{2Y}$pYAA
3	LQF$_{2Y}$DNpYAA
4	ITMDQpYAA
5	WGTDSpYAA
6	SSFDVpYAA
7	SRHEWpYAA
8	ETDFApYAA
9	NDLF$_{2Y}$EpYAA
10	FTSGLpYAA

[a] M, norleucine.

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experiment without the PTP1B treatment resulted in no pink/red colored beads at all (data not shown). Since ~25% of the library members contain at least one F_2Y residue in the random region, the lack of any pink/red beads in the control provides further evidence that F_2Y is completely resistant to tyrosinase action. Most importantly, F_2Y is compatible with every aspect of library synthesis, screening, and sequence identification.

In summary, we have found that F_2Y is a functional, tyrosinase-resistant mimetic of tyrosine for applications such as defining the substrate specificity of PTPs through combinatorial library screening. An efficient method has been developed for the chemoenzymatic synthesis of fully protected F_2Y and its incorporation into peptides and peptide libraries. In addition, an improved PTP screening assay has been developed by employing MBTH as the coupling reagent. Because the pink/red pigment is only formed on the beads that have undergone PTP reaction, the current method does not generate any false positives and is operationally straightforward. Application of the methodologies to systematically profile the substrate specificity of PTPs is currently ongoing in our laboratory and will be reported elsewhere in due course.

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Supporting Information Available: Detailed experimental procedure and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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