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

(Supporting Information)

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Materials. Mushroom tyrosinase (T3824), (+)-biotin hydrazide (B7639), α-cyano-4-hydroxycinnamic acid and organic solvents were obtained from Sigma-Aldrich. Reagents for peptide synthesis were from Advanced ChemTech, Peptides International, and NovaBiochem. N-(9-Fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSU) was from Advanced ChemTech. Protein concentration was determined by the Bradford method using bovine serum albumin (Sigma-Aldrich) as standard. Pyruvic acid and pyridoxal 5'-phosphate (pyridoxal-P) were purchased from United States Biochemical Company. Ammonium acetate was from Fisher Scientific Company. The Dowex-50WX8-400 was obtained from Sigma-Aldrich. PTP1B was expressed in *E. coli* and purified as previously described.[17]

Buffers. Buffer A, 0.1 M KH_2PO_4 (pH 7.2) filtered through a 0.45 μM Acrodisc syringe filter (Life Sciences); buffer B, 100 mM Hepes (pH 7.4), 100 mM NaCl, 2 mM EDTA.

Enzymatic Synthesis of 3,5-Difluorotyrosine (F_2Y). Plasmid pTZTPL,[18] which encodes the tyrosine-phenol lyase (TPL) of *Citrobacter freundii,* was a generous gift from Dr. P. A. Cole (Johns Hopkins University). Recombinant TPL was expressed in *E. coli* and purified as previously described.[18] F_2Y was prepared from 2,6-difluorophenol (Aldrich), ammonia acetate, and pyruvate using tyrosine phenol-lyase, as previously described.[19] The reaction mixture was incubated at room temperature for 14 days in the dark. The reaction was acidified to pH 3 by the addition of acetic acid. The solution was filtered by gravity through a 185-mm Whatman filter paper. The filtrate was loaded onto a column packed with 70 g of pre-washed, according to the manufacturer's instructions, Dowex-50WX8-400. 3,5-Difluorotyrosine was eluted using a 10% NH_4OH solution. The fractions that displayed a high UV absorbance at 276 nm were collected and pooled. The solution was concentrated by rotary evaporation under vacuum and the remaining solution was lyophilized to a solid. Crude 3,5-difluorotyrosine prepared in this manner was a light yellow solid.

***N*-(9-Fluorenylmethoxycarbonyl)-L-3,5-Difluorotyrosine Methyl Ester (2).** F_2Y (**1**) (6.0 g, 27.65 mmol) was dissolved in 10% Na_2CO_3 solution (200 mL), and a solution of Fmoc-OSu (18.65 g, 55.30 mmol) in dioxane (100 mL) was added dropwise with stirring at room temperature. The reaction was continued overnight with stirring. The reaction mixture was poured into water (100 mL) and extracted with diethyl ether (2 x 200 mL). The aqueous layer was acidified to pH 3 by the addition of concentrated HCl with vigorous stirring and extracted with ethyl acetate (2 x 300 mL). The combined organic fractions were pooled and washed with water (100 mL), brine (100 mL), dried over $MgSO_4$, and concentrated under reduced pressure to give Fmoc-3,5-difluorotyrosine, which was used directly in the next reaction step.

To an ice cooled, stirred solution of Fmoc-3,5-difluorotyrosine in methanol (140 mL) was slowly added thionyl chloride (6 mL, 82.95 mmol) over a period of 10 min. The reaction mixture was slowly heated to a reflux and refluxing was continued for 4 h under argon atmosphere. The reaction mixture was cooled to room temperature and the solvent was removed by evaporation under reduced pressure. The reaction mixture was extracted with ethyl acetate (2 x 300 mL). The combined organic fractions were washed with water (100 mL), brine (100 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was recrystallized from dichloromethane to give Fmoc-3,5-difluorotyrosine methyl ester **2** (6.3 g, 50 % yield) as a white solid. R_f 0.39 (40% ethyl acetate in hexane); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.76 (d, J = 7.4 Hz, 2H), 7.55 (t, J = 6.4 Hz, 2H), 7.42-7.36 (m, 2H), 7.34-7.27 (m, 2H), 6.63 (d, J = 7.4 Hz, 2H), 5.25 (d, J = 7.8 Hz, 1H), 4.99 (br s, 1H), 4.60 (m, 1H), 4.47 (m, 1H), 4.37 (m, 1H), 4.20 (t, J = 6.6 Hz, 1H), 3.73 (s, 3H), 3.05-2.92 (m, 2H); ^{19}F NMR (376.5 MHz, $CDCl_3$, proton decoupled): δ -134.99; ^{13}C NMR (100 MHz, DMSO-d_6, proton decoupled): δ 172.1, 155.9, 153.2 (d, J = 8.1 Hz), 150.8 (d, J = 7.0 Hz), 143.7, 143.6, 140.7, 132.1 (t, J = 16.1 Hz), 128.3 (t, J = 8.0 Hz), 127.7, 127.0, 125.2, 125.1, 120.1, 112.6 (d, J = 7.0 Hz), 112.4 (d, J = 7.0 Hz), 65.7, 55.2, 52.0, 46.6, 35.3; HRESI-MS: m/z calcd for $C_{25}H_{21}F_2NO_5Na^+$ (M + Na^+) 476.1285, found 476.1259.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-*tert*-butyl-L-3,5-difluorotyrosine Methyl Ester** (**10**). To a stirred solution of compound **2** (2.00 g, 4.41 mmol) in dichloromethane (100 mL) was added concentrated H_2SO_4 (0.35 mL, 6.62 mmol). The reaction mixture was slowly heated to a reflux and refluxing was continued for 30 min to dissolve most of the starting material. Isobutylene gas was passed through the solution until most of the starting material disappeared as judged by TLC (~6 h). The reaction was quenched by the dropwise addition of 10% $NaHCO_3$ at 0 ^{o}C and stirred for ~3 h at room temperature to evaporate the isobutylene gas. The volatiles were removed under reduced pressure, and the remaining mixture was extracted with ethyl acetate (2 x 150 mL). The combined organic fractions were washed with water (75 mL), brine (75 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography using ethyl acetate/hexane (1:4) to afford the desired product (1.6 g, 71% yield) as a thick syrup. R_f 0.54 (40% ethyl acetate in hexane); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (d, J = 7.4 Hz, 2H), 7.57 (t, J = 6.4 Hz, 2H), 7.39 (t, J = 7.3 Hz, 2H), 7.34-7.27 (m, 2H), 6.66 (d, J = 7.9 Hz, 2H), 5.44 (d, J = 7.9 Hz, 1H), 4.63 (m, 1H), 4.45 (m, 1H), 4.36 (m, 1H), 4.20 (t, J = 6.8 Hz, 1H), 3.71 (s, 3H), 3.05 (dd, J = 5.6, 13.9 Hz, 1H), 2. 98 (dd, J = 6.2, 13.9 Hz, 1H), 1.36 (s, 9H); ^{19}F NMR (376.5 MHz, $CDCl_3$, proton decoupled): δ -122.47; ^{13}C NMR (100 MHz, $CDCl_3$, proton decoupled): δ 171.4, 159.0 (d, J = 6.0 Hz), 156.6 (d, J = 6.0 Hz), 155.4, 143.7, 143.5, 141.3, 132.1 (t, J = 9.1 Hz), 130.8 (t, J = 16.1 Hz), 127.7, 127.0, 126.9, 125.0, 124.9, 119.9, 112.8 (d, J = 6.0 Hz), 112.6 (d, J = 6.0 Hz), 82.9, 66.9, 54.5, 52.4, 47.1, 37.6, 28.2; HRESI-MS: m/z calcd for $C_{29}H_{29}F_2NO_5Na^+$ (M + Na^+) 532.1911, found 532.1900.

***N*-(9-Fluorenylmethoxycarbonyl)-*O*-*tert*-butyl-L-3,5-difluorotyrosine** (**3**). To an ice cooled, stirred solution of compound **10** (1.00 g, 1.96 mmol) in THF (12 mL) and H_2O (12 mL) was added LiOH (52 mg, 2.16 mmol). The reaction mixture was allowed to warm up to room temperature and continued with stirring for 1 h. The reaction mixture was then washed with diethyl ether (2 x 20 mL) to remove any byproducts. The aqueous layer was acidified to pH 4 with concentrated HCl and extracted with ethyl acetate (2 x 50 mL). The organic fraction was washed with water (30 mL) and brine (30 mL), and then dried over $MgSO_4$. The solvent was

removed under reduced pressure to give the target compound **3** as a white solid (0.65 g, 67 %). R_f 0.21 (40% ethyl acetate in hexane); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (d, J = 7.4 Hz, 2H), 7.58-7.50 (m, 2H), 7.38 (t, J = 7.4 Hz, 2H), 7.33-7.26 (m, 2H), 6.69 (d, J = 7.7 Hz, 2H), 5.22 (d, J = 7.6 Hz, 1H), 4.65 (m, 1H), 4.45 (m, 1H), 4.37 (m, 1H), 4.19 (t, J = 6.6 Hz, 1H), 3.11 (dd, J = 4.8, 13.8 Hz, 1H), 3.00 (dd, J = 5.4, 13.8 Hz, 1H), 1.34 (s, 1H); ^{19}F NMR (376.5 MHz, $CDCl_3$, proton decoupled): δ -122.21; ^{13}C NMR (100 MHz, $CDCl_3$, proton decoupled): δ 175.3, 159.1 (d, J = 6.6 Hz), 156.6 (d, J = 6.6 Hz), 155.8, 143.7, 143.5, 141.2, 132.1 (t, J = 7.8 Hz), 130.8 (t, J = 16.8 Hz), 127.7, 127.1, 125.0, 124.9, 120.0, 112.9 (d, J = 5.8 Hz), 112.7 (d, J = 5.8 Hz), 83.1, 67.2, 54.3, 47.0, 37.2, 28.3; HRESI-MS: m/z calcd for $C_{28}H_{27}F_2NO_5Na^+$ (M + Na^+) 518.1755, found 518.1729.

Synthesis of F_2Y-Containing Peptides. Individual peptides were synthesized on 100 mg of CLEAR-amide resin using standard Fmoc/HBTU/HOBt chemistry. For the coupling reaction of F_2Y and pY, 1.5 and 2.0 equivalents of Fmoc-amino acids, respectively, were employed, whereas 4.0 equivalents were used for all other amino acids. The resin-bound peptides were washed with dichloromethane and cleaved from the resin and side-chain deprotected using a modified reagent K [7.5% phenol, 5% water, 5% thioanisole, 2.5% ethanedithiol and 1% anisole in trifluoroacetic acid (TFA)] at room temperature for 60 min. After evaporation of solvents, the mixture was triturated three times with 20 volumes of cold Et_2O. The precipitate was collected and dried under vacuum. The crude peptides were purified by reversed-phase HPLC on a semi-preparative C_{18} column. The identity of each peptide was confirmed by MALDI-TOF mass spectrometric analysis.

PTP Assay. PTP assays were performed with the synthetic pY peptides as substrates in a quartz microcuvette. A typical reaction (total volume 120 μL) contained buffer B, 1 mM TCEP, 0.1 mg/mL bovine serum albumin and 0–80 μM pY peptide. The reaction was initiated by the addition of 10 μL of a PTP1B stock solution (final concentration 10 nM) and monitored continuously at 282 nm ($\Delta\varepsilon$ = 1102 M^{-1} cm^{-1}) on a UV-VIS spectrophotometer. The initial rates were calculated from the early regions of the reaction progress curves (<60 s) and fitted to the Michaelis-Menten equation to obtain the k_{cat}, K_M and k_{cat}/K_M values for each peptide. Three independent sets of measurements were performed for each peptide and the data reported represent the mean ± SD from the triplicate experiments.

Derivatization of Tyr- and F_2Y-Containing Peptides by Biotin Hydrazide. Crude peptide REYEFpYAA (final concentration 500 μM) in 0.1 M K_2HPO_4 (pH 7.4) was treated with tyrosinase (final concentration 1.0 μM) and biotin hydrazide (final concentration 2.0 mM). The mixture was incubated at room temperature for 1 h and passed through a C_{18} cartridge (Burdick and Jackson BαJ solid phase System columns) to remove the protein. The resulting solution was analyzed by reversed-phase HPLC on a C_{18} column (Vydac 300 Å, 4.6 x 250 mm). The column was eluted with a linear of gradient of 10–60% acetonitrile in water containing 0.05% TFA (flow rate 1.0 mL/min). The fractions corresponding to major elution peaks were analyzed by MALDI-TOF mass spectrometry (Bruker Reflex III). The experimental condition was identical for peptide REF_{2Y}EFpYAA, except that the HPLC gradient contained 0−50% CH_3CN.

The crude, untreated peptide REYEFpYAA gave two major peaks on HPLC (Figure S1). The peak at retention time (T_R) of 32.0 min produced a single peak of m/z 1168.4 in MALDI MS

and thus contained the desired peptide. The second peak (at 36.7 min) contained no contents with m/z >700. After treatment with tyrosinase and biotin hydrazide, the peak at 32.0 min completely disappeared and a new peak appeared at 34.5 min. The MALDI MS of the new species showed two peaks at m/z 1438.7 and 1440.7. The m/z 1440.7 peak corresponds to the peptide derivatized with a single biotin hydrazide molecule (Figure S1), whereas the m/z 1438.7 was likely due to further oxidation of the above adduct, resulting in the loss of two protons.

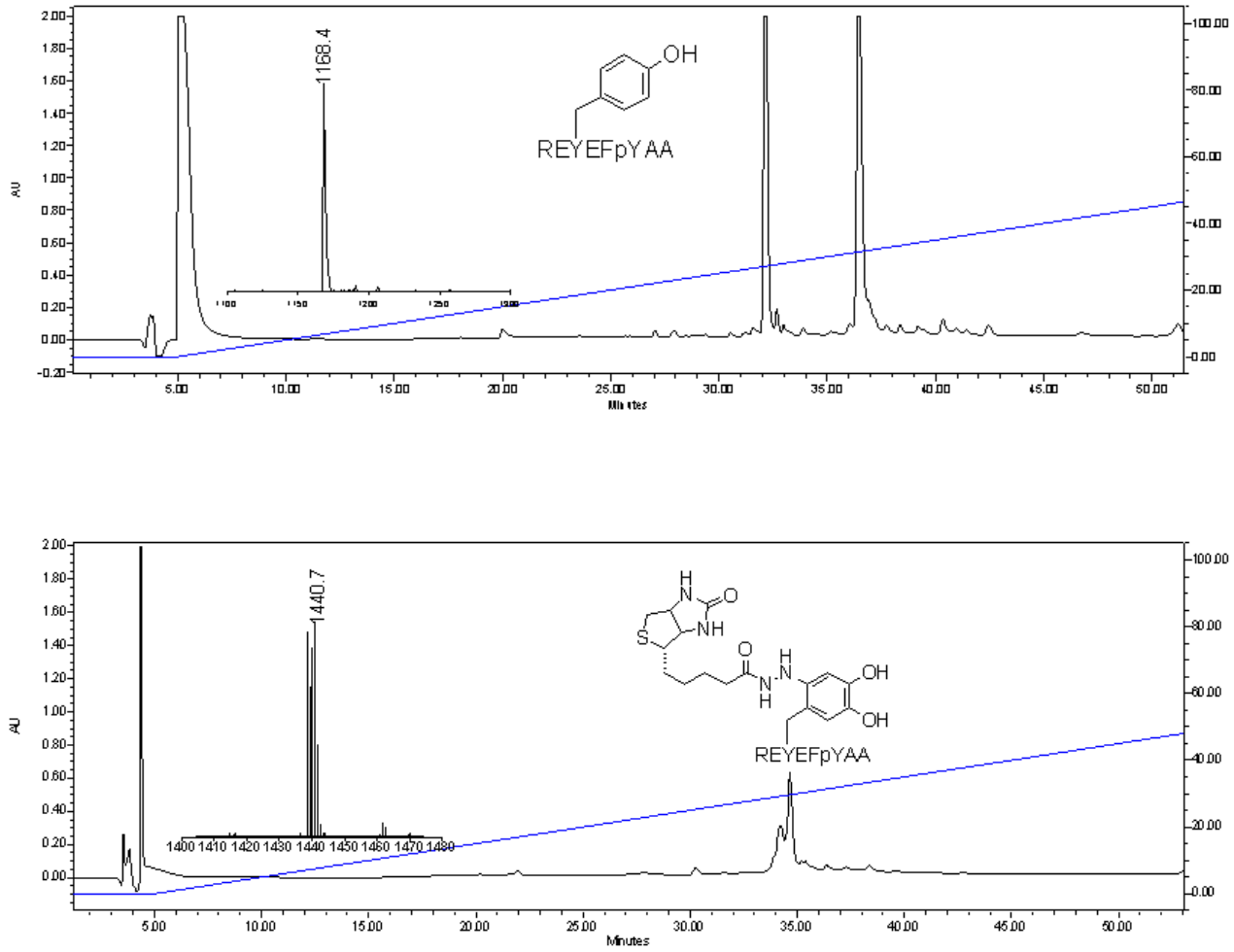


Figure S1. HPLC analysis of untreated and treated peptide REYEFpYAA.

Figure S2 shows the HPLC tracings of untreated (A) and treated peptide $REF_{2Y}EFpYAA$ (B). The peak at T_R = 33.9 min, which was unchanged after tyrosinase treatment, gave a single peak of m/z 1204.6 in MALDI-MS, corresponding to the unreacted peptide $REF_{2Y}EFpYAA$. The peak at 38.8 min was due to a low-molecular-weight impurity (which gave no signal at m/z >700 in MALDI MS). The peak at T_R = 22.0 min in Figure 2SB was from the excess biotin hydrazide in the reaction mixture.

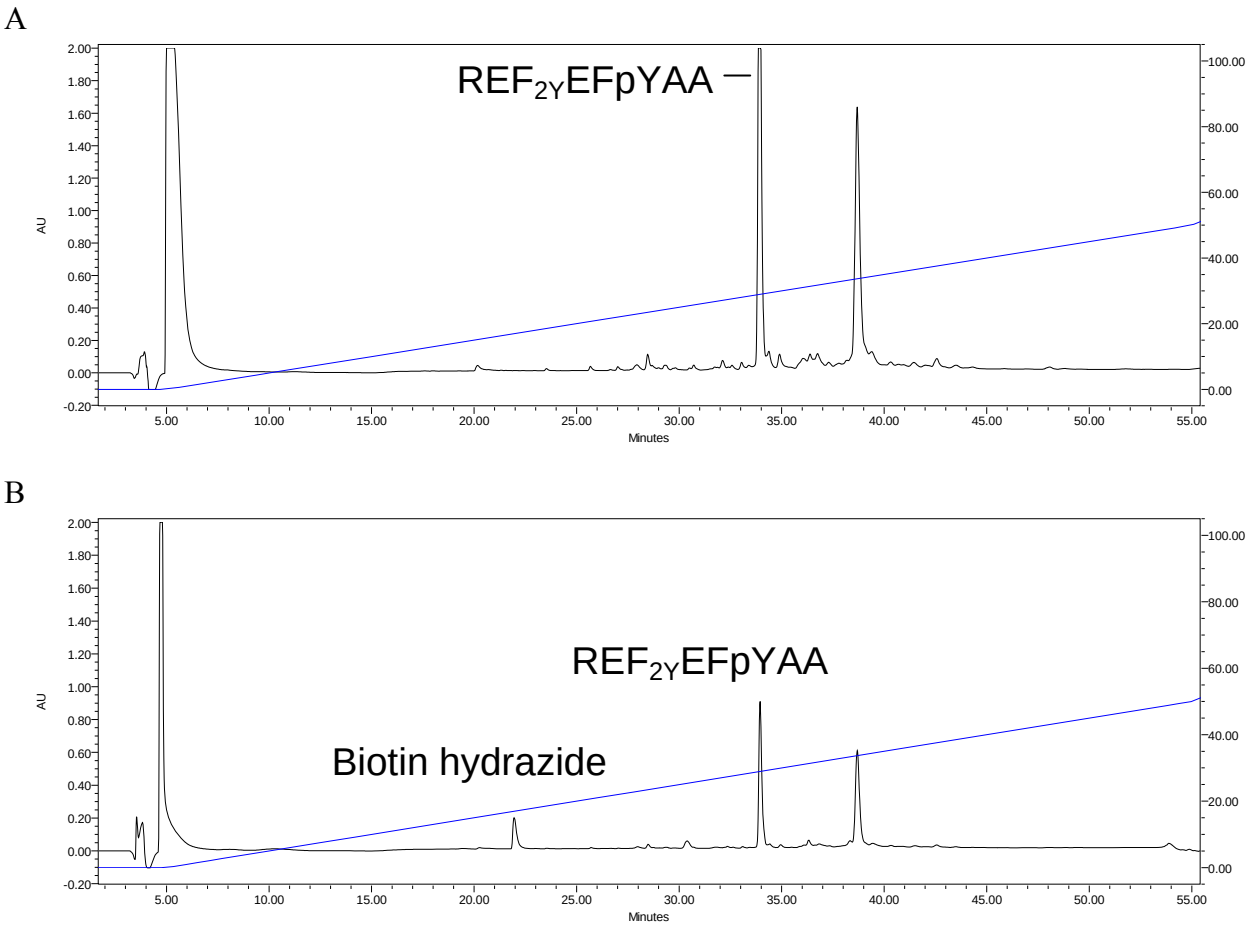


Figure S2. HPLC analysis of untreated (A) and treated peptide $REF_{2Y}EFpYAA$ (B).

Synthesis of F_2Y-Containing Peptide Library. Peptide library (SAXXXXXpYAABBRM-resin, where B is β-alanine and X is F_2Y, norleucine, or any of the 17 proteinogenic amino acids excluding Met, Cys and Tyr) was synthesized on 5 g of amino PEGA resin (50-100 μm, 0.4 mmol/g). All of the manipulations were performed at room temperature unless otherwise noted. The invariant positions (pYAABBRM and SA) were synthesized with 4 equivalents of Fmoc-amino acids using HBTU/HOBt/N-methylmorpholine (NMM) as the coupling reagents and the coupling reaction was terminated after ninhydrin tests were negative. For the synthesis of random residues, the resin was split into 19 equal portions and each portion was coupled twice, each with 5 equiv of a different Fmoc-amino acid/HBTU/HOBt/NMM for 1 h (except for Fmoc-F_2Y, for which 2.5 equivalents were used and coupled only once). To facilitate sequence determination by mass spectrometry, 5% (mol/mol) CD_3CO_2D was added to the coupling reactions of leucine and lysine, whereas 5% $CH_3CD_2CO_2D$ was added to the coupling reaction of Nle.[20] The resin-bound library was washed with dichloromethane and side-chain deprotected using the modified reagent K at room temperature for 1.5 h. The library was washed with TFA, DCM and DMF and stored in DMF at 4 °C until use.

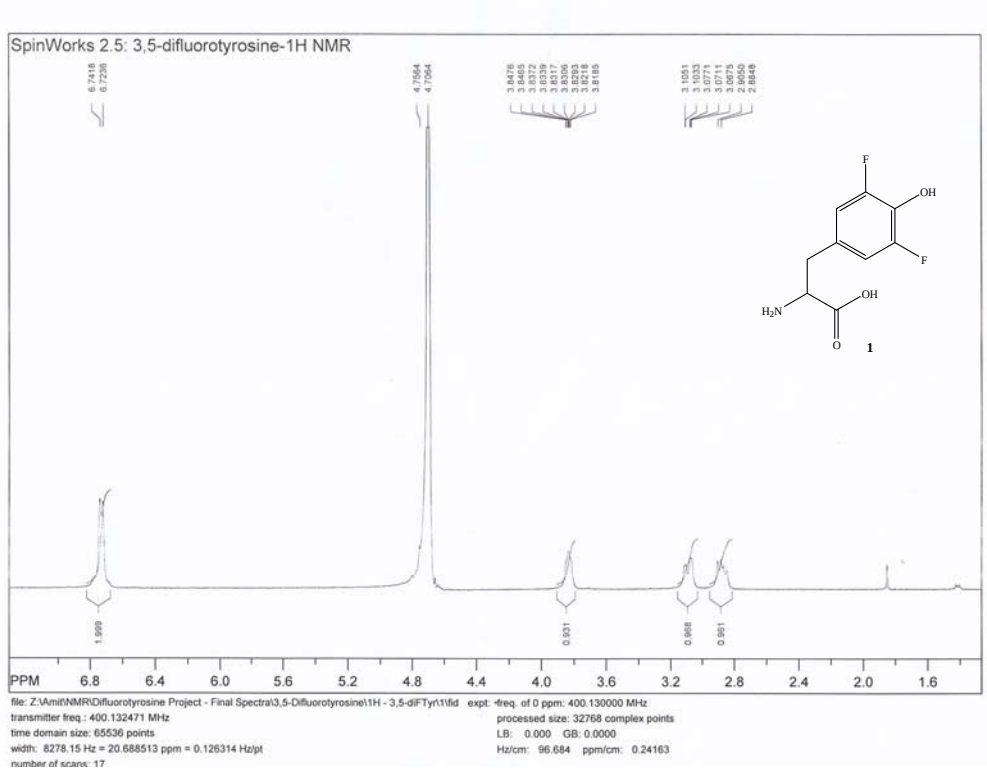
Library Screening. In a typical screening experiment, 200 mg of the library (wet resin) in DMF was placed in a micro-BioSpin column (0.8 mL, Bio-Rad) and extensively washed with

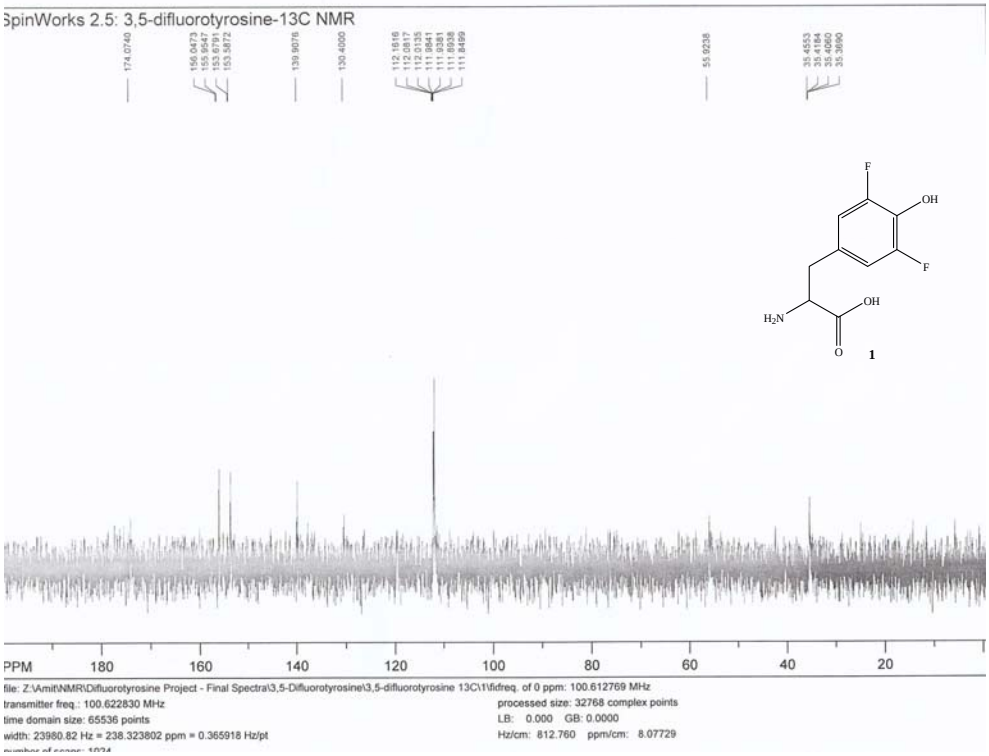
DMF and ddH_2O. The resin was then blocked for 1 h with blocking buffer (30 mM Hepes, pH 7.4, 150 mM NaCl, 0.01% Tween 20, and 0.1% gelatin). The library was incubated with 1.0 nM PTP1B in 50 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM EDTA, 5 mM TCEP at room temperature for 20 min with gentle mixing. The resin was drained and resuspended in 0.1 M KH_2PO_4 (pH 6.8) containing 1.2 μM mushroom tyrosinase and 6 mM 3-methyl-2-benzothiazolinonehydrazone (MBTH). The resulting mixture was incubated at room temperature with gentle mixing and exposure to air. Pink/red beads typically appeared after 20 min and were isolated manually under a dissecting microscope and sequenced by PED/MS.[20] Control experiments without PTP1B produced no colored beads under identical conditions.

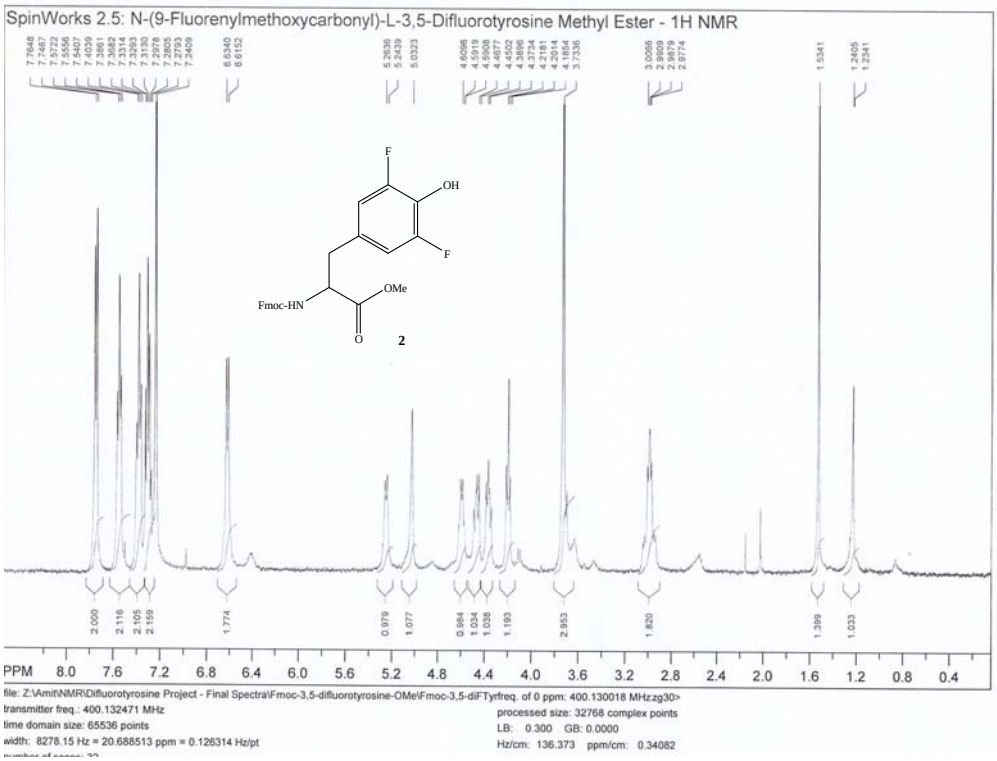
References:

(17) Park, J.; Pei, D. *Biochemistry* **2004**, *43*, 15014.
(18) Chen, H., Demidkina, T.V. and Phillips, R.S. *Biochemistry* **1990**, *34*, 12276-12283.
(19) Phillips, R. S., Fletcher, J. G., Von Tersch, R. L. and Kirk, K. L. *Arch. Biochem. Biophys.*, **1990**, *276*, 65–69
(20) Thakkar, A.; Wavreille, A.-S.; Pei, D. *Anal. Chem*. **2006**, *78*, 5935−5938.

^{1}H, ^{13}C, and ^{19}F spectra for Compounds **1**, **2**, **10**, and **3**:



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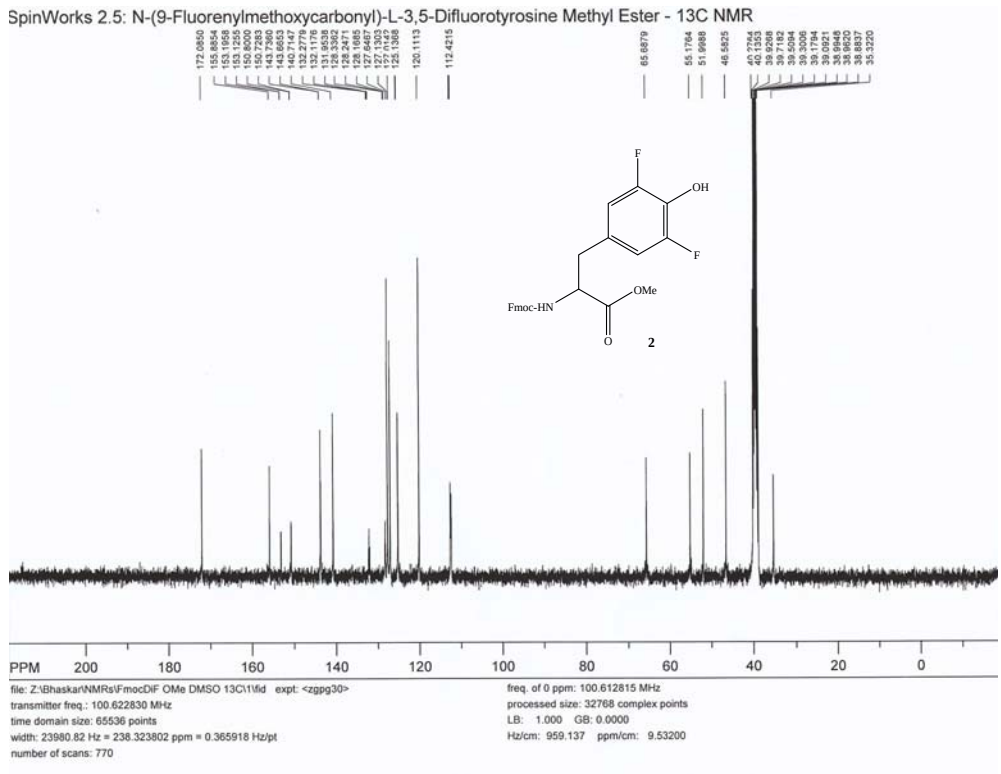
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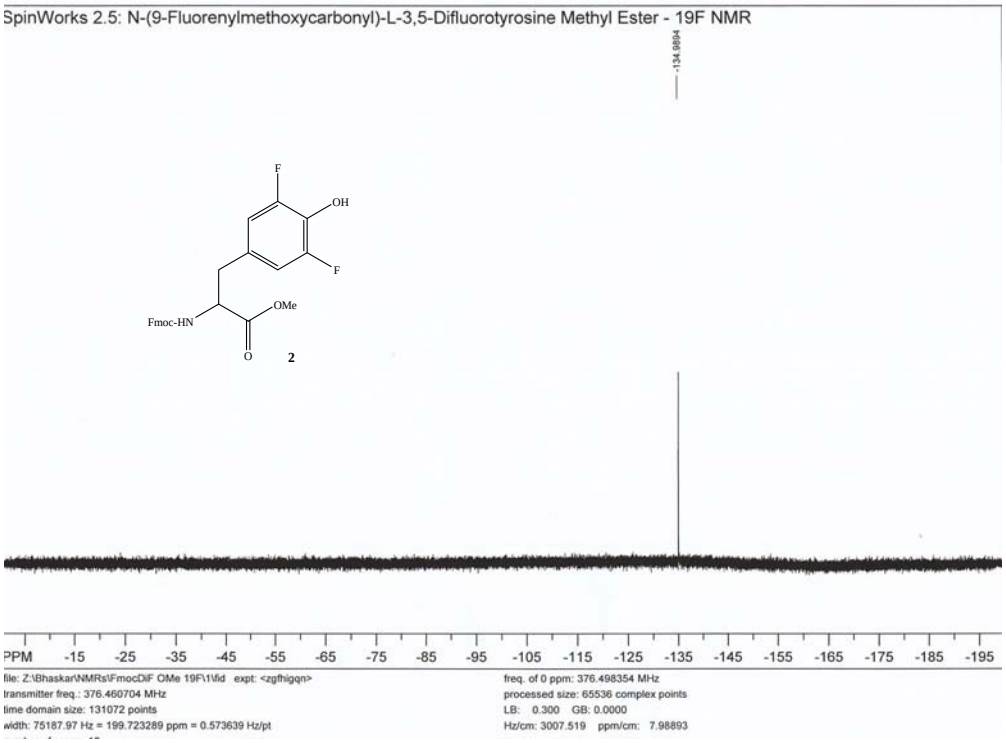

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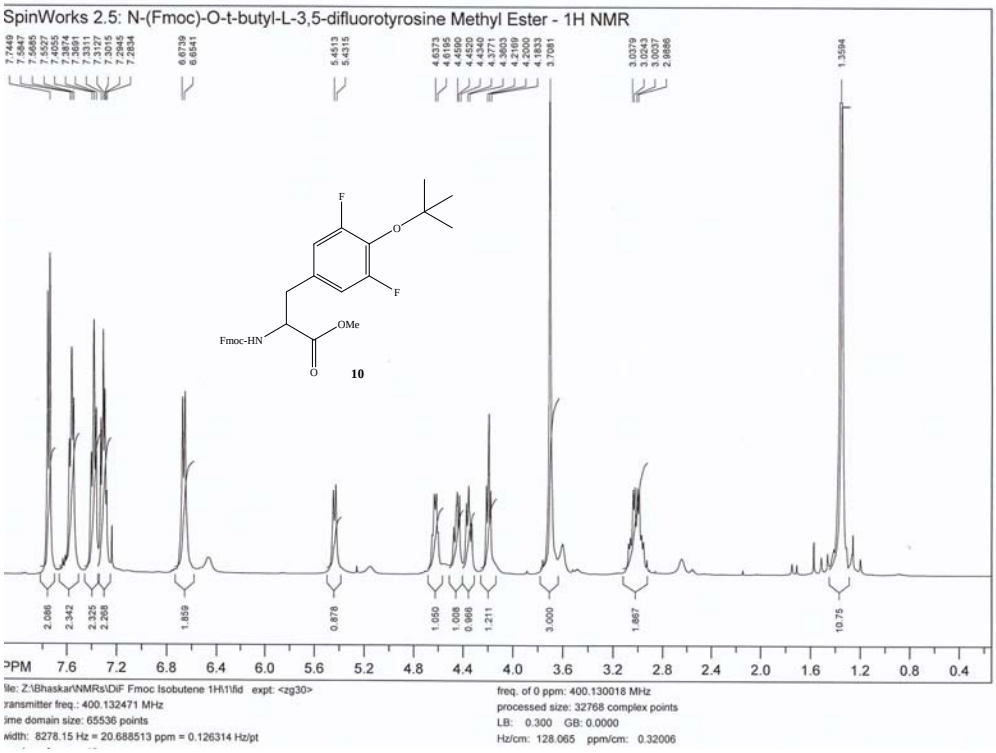

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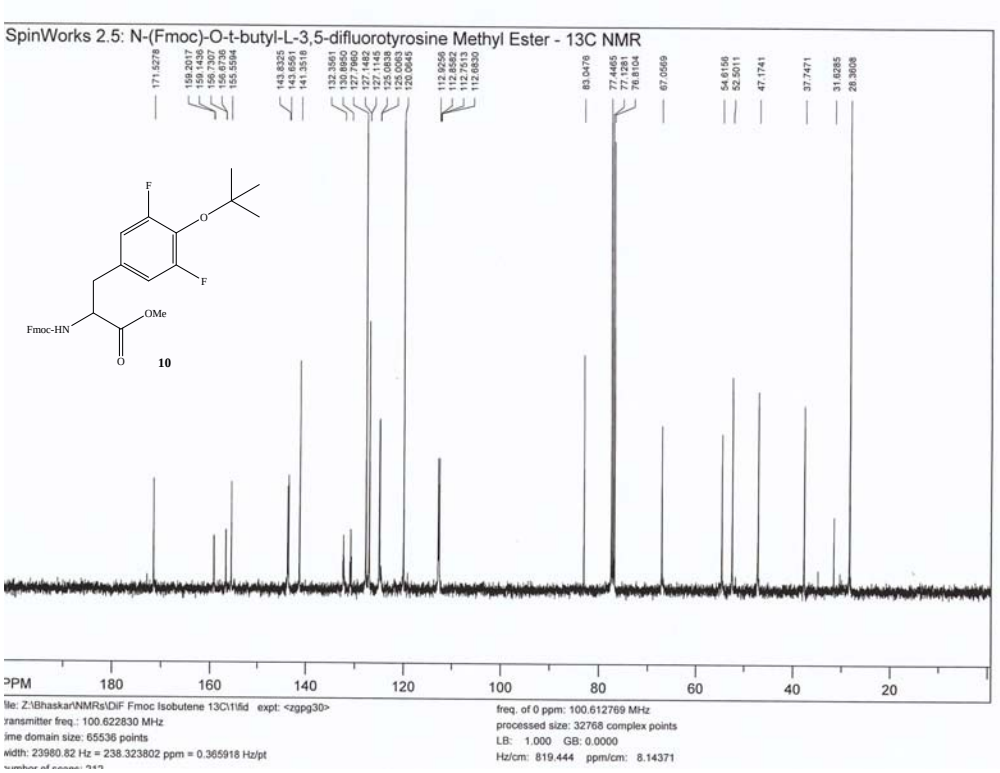
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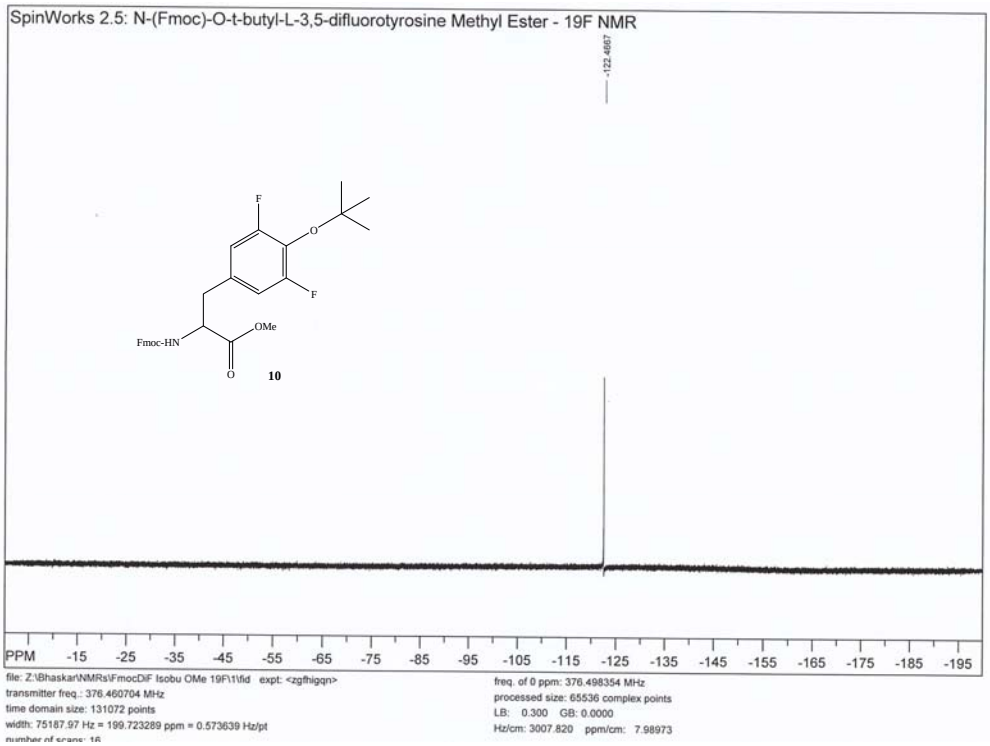
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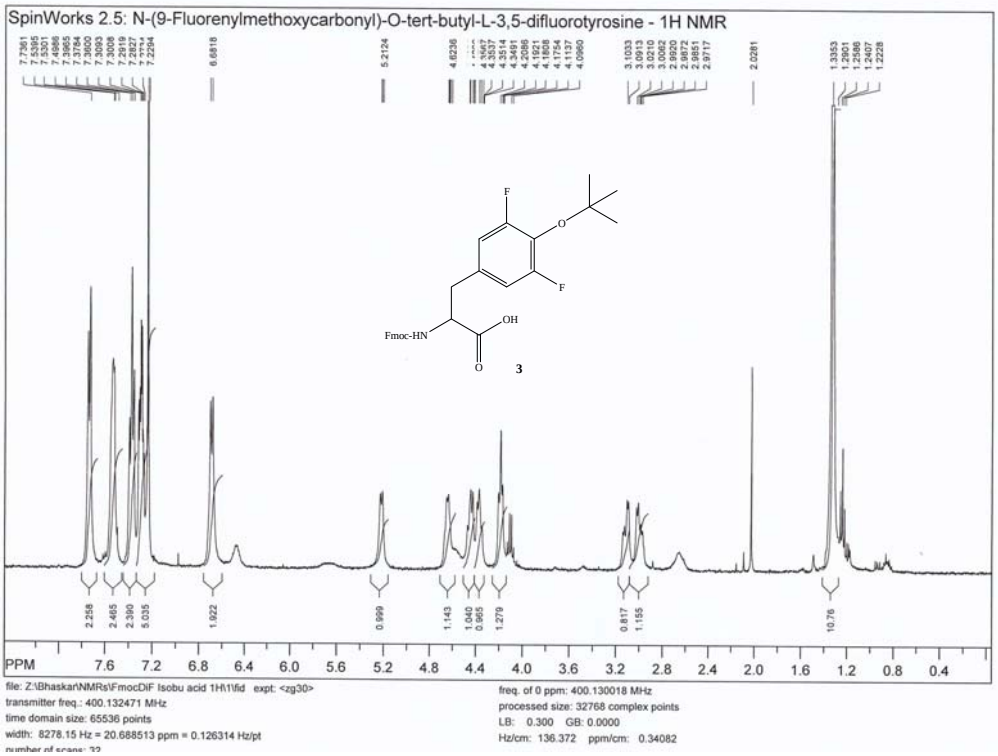
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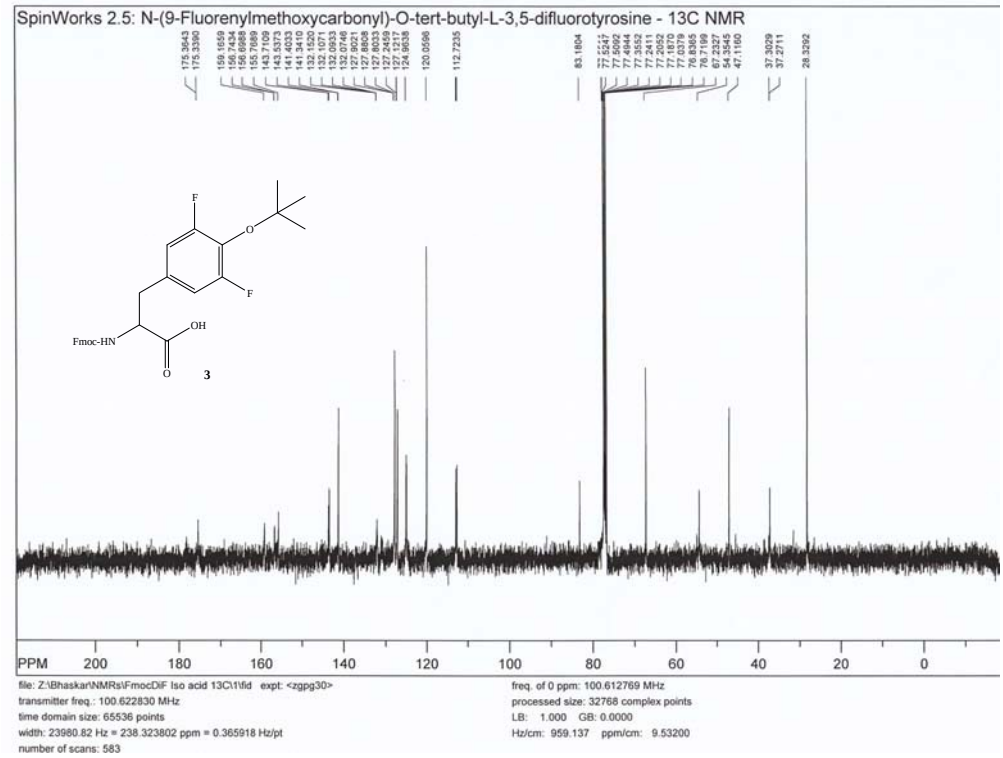
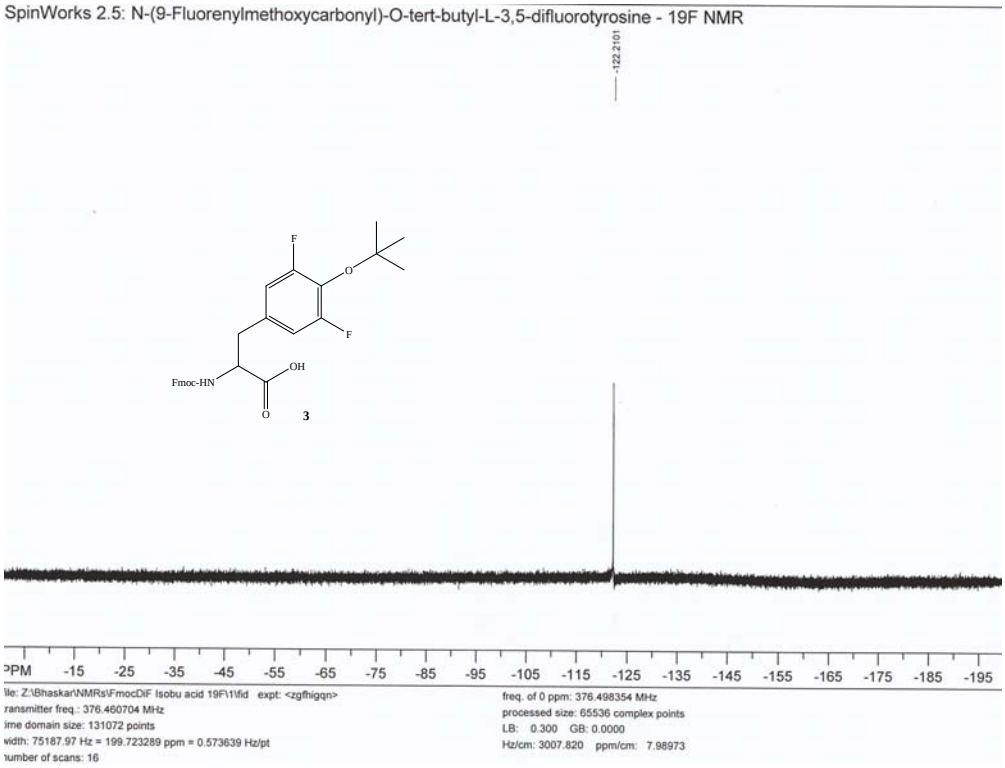
Hz/cm: 136.372 ppm/cm: 0.34082


SpinWorks 2.5: N-(9-Fluorenylmethoxycarbonyl)-O-tert-butyl-L-3,5-difluorotyrosine - 13C NMR
F
O
F
Fmoc-HN
OH
O
3
PPM
file: Z:\Bhaskar\NMRs\FmocDiF Iso acid 13C\1\fid expt: <zgpg30>
transmitter freq.: 100.622830 MHz
time domain size: 65536 points
width: 23980.82 Hz = 238.323802 ppm = 0.365918 Hz/pt
number of scans: 583
freq. of 0 ppm: 100.612769 MHz
processed size: 32768 complex points
LB: 1.000 GB: 0.0000
Hz/cm: 959.137 ppm/cm: 9.53200

SpinWorks 2.5: N-(9-Fluorenylmethoxycarbonyl)-O-tert-butyl-L-3,5-difluorotyrosine - 19F NMR



File: Z:\Bhaskar\NMRs\FmocDiF Isobu acid 19F\1\fid expt: <zgflqn>
Transmitter freq.: 376.460704 MHz
Time domain size: 131072 points
Width: 75187.97 Hz = 199.723289 ppm = 0.573639 Hz/pt
Number of scans: 16

freq. of 0 ppm: 376.498354 MHz
processed size: 65536 complex points
LB: 0.300 GB: 0.0000
Hz/cm: 3007.820 ppm/cm: 7.98973