Supplementary Material

Site-Specific Orthogonal Labeling of the Carboxy Terminus of α-Tubulin †

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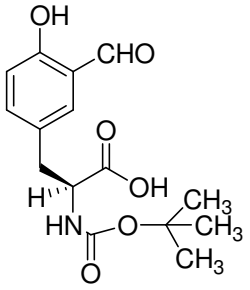
Materials:

NBD-Cl, salicylaldehyde, and deuterated solvents (DMSO, d-6; D_2O and $CDCl_3$) were obtained from Acros organics and used as received. Deuterated sodium hydroxide (NaOD) was made by adding sodium metal to D_2O. SDS (sodium dodecyl sulfate, catalog # L5750), subtilisin Carlsberg (catalog # P5380), monoclonal anti-tyrosine tubulin antibody (TUB-1A2), and hydrazine monohydrate were obtained from Sigma-Aldrich. Secondary antibody (goat anti-mouse IgG H+L HRP conjugate) and tetramethylbenzidine (TMB) were obtained from Invitrogen. OxyBlot™ protein detection oxidation kit was obtained from Chemicon International (catalog # S7150). All other chemicals were reagent grade or better and used without further purification.

Synthesis:

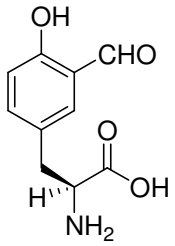


1

(S)-2-(tert-Butoxycarbonylamino)-3-(3-formyl-4-hydroxyphenyl)propanoic acid (1)

A solution containing (S)-2-(tert-butoxycarbonylamino)-3-(4-hydroxyphenyl)-propanoic acid (BOC-L-tyrosine) (14 g, 50 mmol), chloroform (51 ml, 630 mmol), anhydrous NaOH (10 g, 250 mmol) and water (1.8 ml, 100 mmol) was heated to 50 °C and stirred for 1 hr. Powdered NaOH (6 g, 150 mmol) was added to the reaction mixture and stirring was continued for 4 hr at 58 °C (*1*).

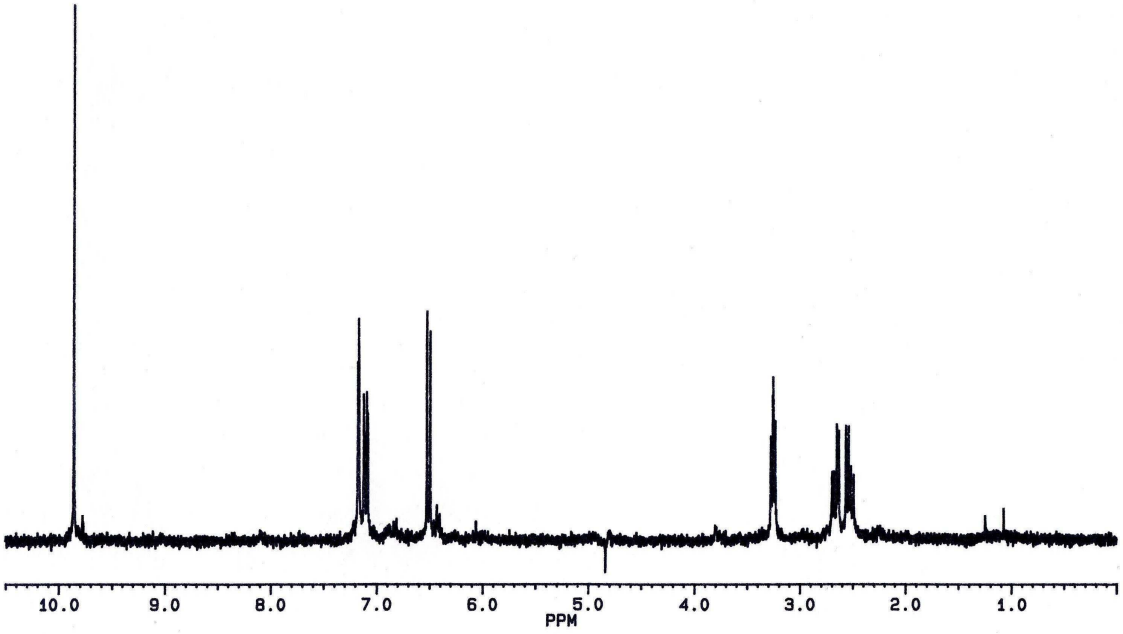
The reaction mixture was acidified with HCl to pH 1 and extracted with ethyl acetate. The ethyl acetate extracts were washed with saturated sodium chloride, dried over magnesium sulfate and concentrated under reduced pressure to produce brown colored oil, which became a brown powder upon vacuum drying. Both starting material and the product were visible in the TLC (8:1 chloroform: methanol, 1 % acetic acid). Pure product was isolated by silica gel chromatography using chloroform as the eluent. The ^{1}H and ^{13}C NMR spectra matched the literature (*2*).



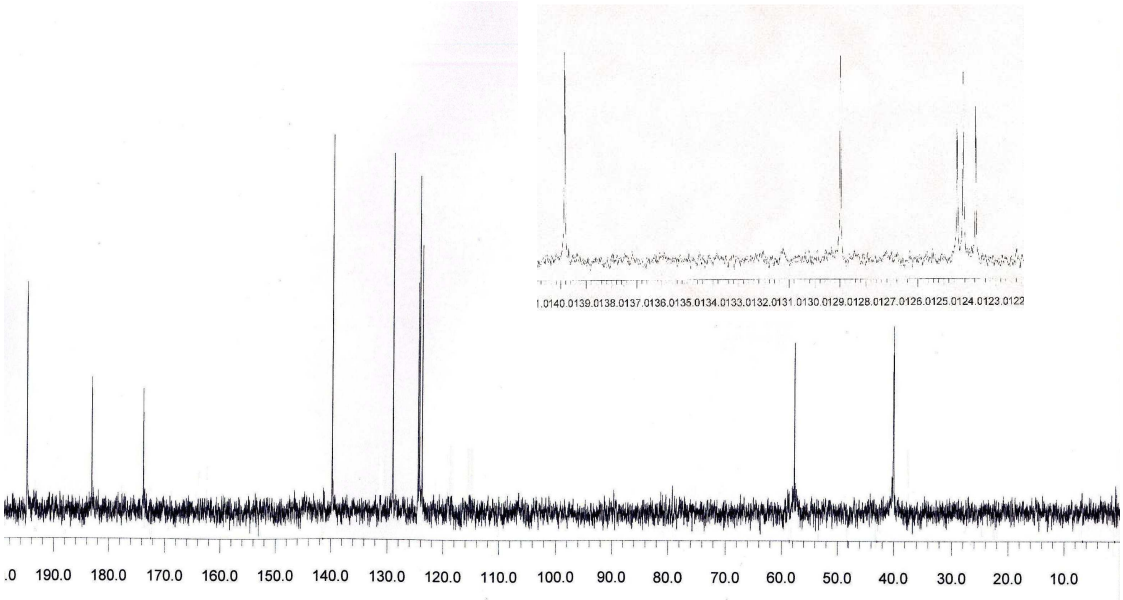
2

(S)-2-Amino-3-(3-formyl-4-hydroxyphenyl)propanoic acid (3-formyltyrosine) (2):

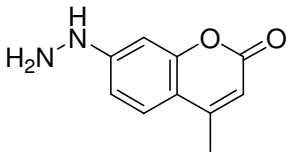
Compound **1** (0.14 g, 0.45 mmol) was dissolved in 1 ml of dichloromethane and cooled in ice. Trifluoroacetic acid (1 ml) was added and the mixture was allowed to reach at room temperature and was stirred for 2 hr. Evaporation of the solvent produced a brown colored oil, which solidified after vacuum drying. ^{1}H NMR (300 MHz, NaOD): δ 2.48 (unresolved m, 1H), 2.68 (unresolved m, 1H), 3.25 (t, 1H, J = 6.28), 6.50 (d, 1H, J = 8.70 Hz), 7.10 (d, 1H, J = 8.75 Hz), 7.16 (s, 1H), 9.85 (s, 1H).^{13}C NMR (360 MHz, NaOD): δ 40.2, 57.7, 123.8, 124.2, 124.5, 127, 140.0, 173.8, 183.1, 194.8.



^{1}H NMR [(S)-2-Amino-3-(3-formyl-4-hydroxyphenyl)propanoic acid (**2**)]



^{13}C NMR [(S)-2-Amino-3-(3-formyl-4-hydroxyphenyl)propanoic acid (**2**)]

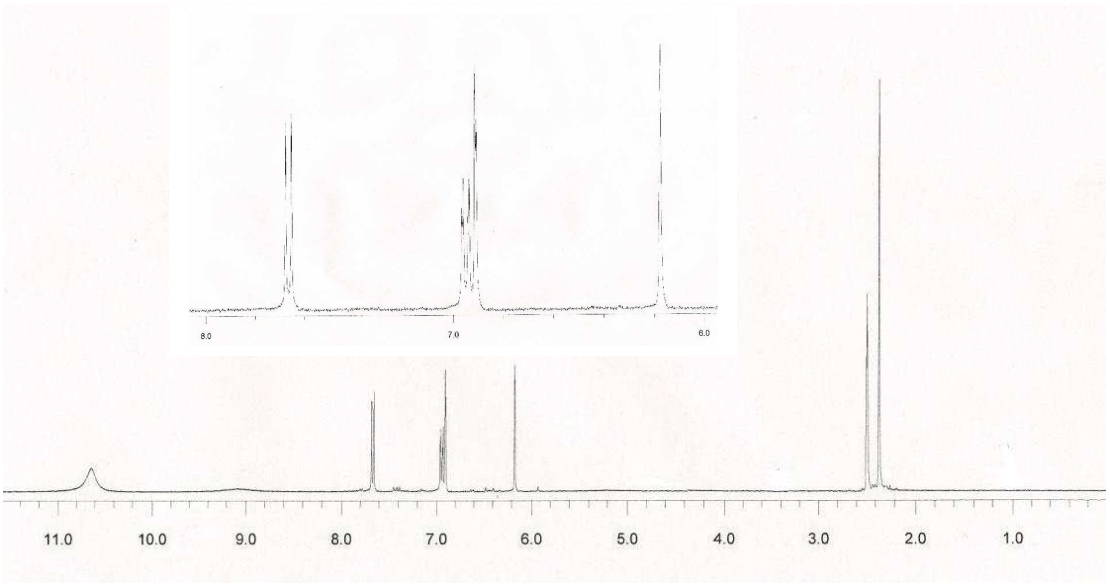


3

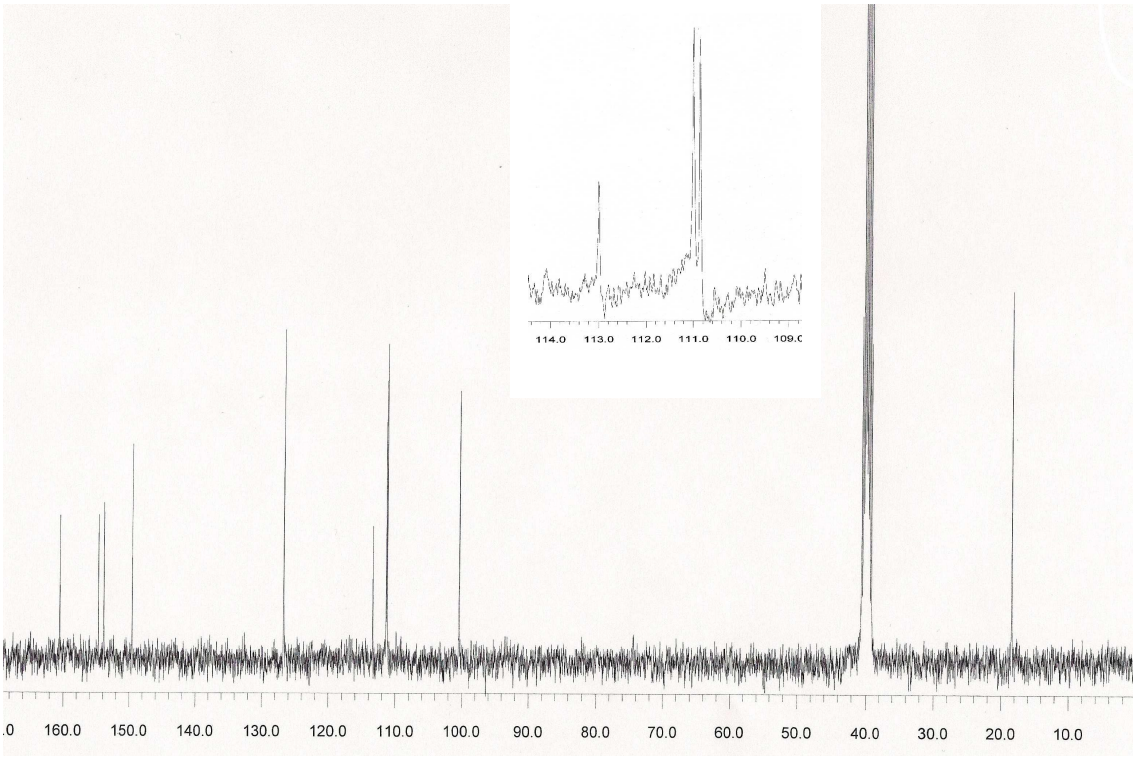
7-Hydrazinyl-4-methyl-2H-chromen-2-one (3)

The starting material 7-amino-4-methyl-2H-chromen-2-one was synthesized according to a literature procedure (*3*).

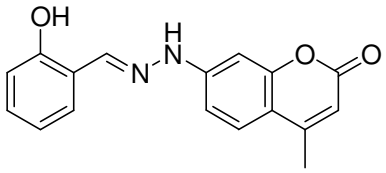
To a stirred solution of 7-amino-4-methyl-2H-chromen-2-one (1.77 g, 10 mmol) in 5 ml of concentrated HCl solution, a solution of 0.8 g (12 mmol) of sodium nitrite in 3 ml of water was added dropwise. The temperature of the reaction mixture was maintained between -5 °C and 0 °C. After stirring for 1 h at 0 °C, the reaction mixture was poured in a thin stream into a cold solution of stannous chloride dihydrate (7.2 g, 32 mmol) in 7.2 ml concentrated HCl. Stirring was continued for 1 h at or below 0 °C (*4*). A light yellow precipitate was collected by vacuum filtration, washed with cold water, cold alcohol and finally with cold diethyl ether and dried to yield 1.5 g (78 %) of product. ^{1}H NMR (DMSO-d_6): δ 2.38 (s, 3H), 6.18 (s, 1H), 6.91 (s, 1H), 6.95 (d, 1H, J = 8.53 Hz), 7.66 (d, 1H, J = 8.57 Hz), 10.65 (broad s, NH). ^{13}C NMR (DMSO-d_6): δ 18.5, 100.4, 111.2, 111.4, 113.4, 126.7, 149.6, 153.9, 154.7, 160.5.



^{1}H NMR [7-hydrazinyl-4-methyl-2H-chromen-2-one (**3**)]



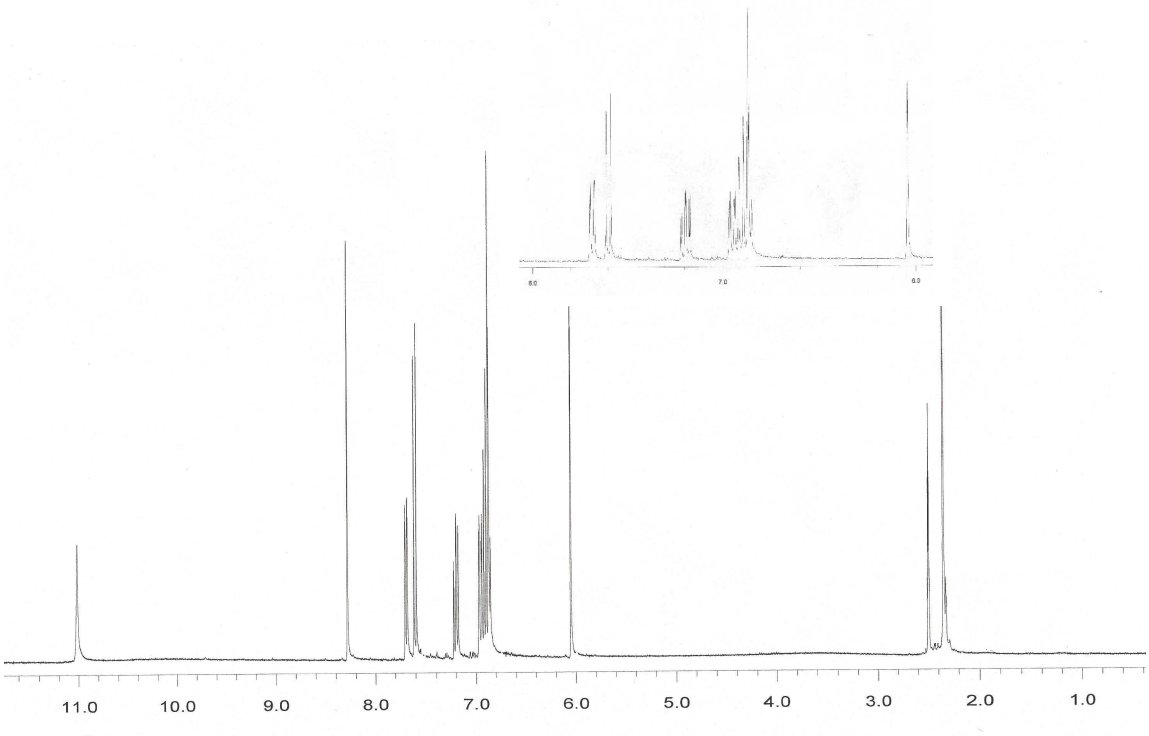
^{13}C NMR [7-hydrazinyl-4-methyl-2H-chromen-2-one (**3**)]



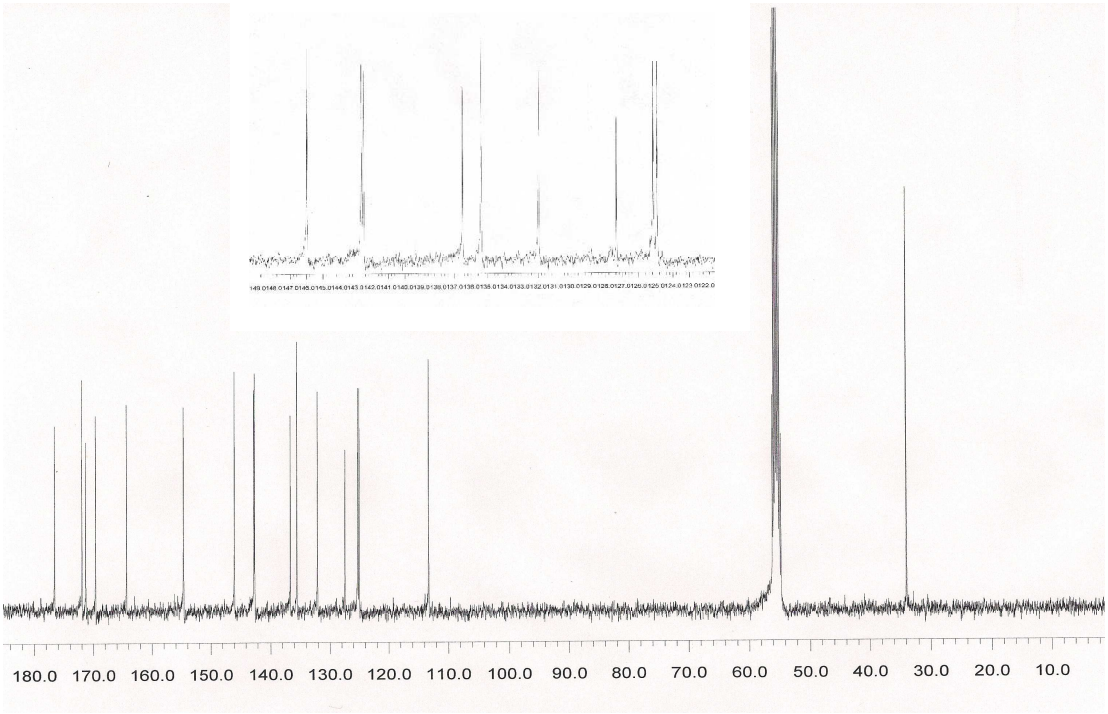
4

7-(2-(2-Hydroxybenzylidene)hydrazinyl)-4-methyl-2H-chromen-2-one (4).

Salicylaldehyde (1.2 g, 10 mmol) in 3 ml of methanol was added to a solution of coumarin hydrazine (compound **3**, 0.19 g, 1 mmol) in 5 ml methanol containing a few drops of trifluoroacetic acid. The solution was stirred at room temperature for 30 min. A yellow precipitate was formed which was filtered, washed and dried to yield 0.15 g (50 %) of the hydrazone. ^{1}H NMR (DMSO-d_6): δ 2.33 (s, 3H), 6.05 (s, 1H), 6.87 (s, 1H), 6.87 (t, 1H, J = 7.20), 6.90 (d, 1H, J = 7.27 Hz), 6.95 (d, 1H, J = 8.75 Hz), 7.20 (t, 1H, J = 8.44), 7.60 (d, 1H, J = 8.75 Hz), 7.69 (d, 1H, J = 7.67 Hz), 8.27 (s, 1H), 11.0 (s, NH or OH). ^{13}C NMR (DMSO-d_6): δ 19.0, 98.3, 109.9, 110.2, 112.3, 117.0, 120.4, 121.5, 127.5, 127.6, 131.0, 139.5, 149.2, 154.5, 156.1, 156.7 and 161.4.



^{1}H NMR [7-(2-(2-Hydroxybenzylidene)hydrazinyl)-4-methyl-2H-chromen-2-one (**4**)]



^{13}C NMR [7-(2-(2-Hydroxybenzylidene)hydrazinyl)-4-methyl-2H-chromen-2-one (**4**)]

TTL expression and purification

The expression vector pReceiver 05x (Genecopoeia, Maryland) was transformed into the *Escherichia coli* expression strain BL21 and grown on 100 µg/ml Ampicillin containing LB (LB-Amp) Agar. The plasmid was re-isolated, restriction digested using Eco RI and Xho I (NEB) and the fragments were subjected to agarose gel electrophoresis to confirm transformation. The transformed BL21 cells were harvested overnight in LB-Amp medium at 37° C. The overnight grown culture was added to fresh LB-Amp medium in 1:10 ratio and incubated at 37° C. At OD_{600} = 0.6 the cells were induced using 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and allowed to grow at 37° C for 20 hours. The cells were centrifuged at 3000 rpm for 20 min at 4° C. The cell pellets were stored at -50° C until used for protein purification. The cell pellets were resuspended in TE buffer (10mM Tris, 1mM EDTA, pH 8) containing 0.3 mg of PMSF(phenylmethanesulphonylfluoride) per ml. The resuspended pellets were sonicated for 10 seconds with 6 watts of output for 6 cycles using a Sonic Dismembrator Model 100 (Fisher) followed each time by 15 seconds incubation on ice. The lysates were then centrifuged at 3000 rpm for 20 min to remove the debris. The supernatant fractions (cleared lysate) were subjected to affinity chromatography. Glutathione Sepharose 4B (GE Healthcare) was washed with PBS (140 mM NaCl, 2.7 mM KCl,10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.4) following manufacturer's instructions. The cleared lysate was incubated with Glutathione Sepharose 4B in 50 ml centrifuge tubes on a rocker at 4° C for 45 min. The fusion protein bound to the resin was washed 5 times with PBS by centrifuging at 1500 rpm for 3 min. The resin was then poured into a 5 ml column and eluted with elution buffer (50 mM Tris-HCl, 20 mM reduced glutathione, pH 8.0). The

elution aliquots were subjected to SDS-PAGE and protein concentration was determined by BCA assay and densitometry.

Note that the GST portion of the expressed protein was not removed prior to use. The expressed TTL obtained from the Wehland lab also contained the GST tag.

Immunodetection of tubulin labeled with 3-formyltyrosine

The OxyBlot™ kit was used for immunodetection of the presence of an aldehyde group (3-formyltyrosine) tubulin (*5*). Bovine brain tubulin was detyrosinated with carboxypeptidase A followed by retyrosination with 3-formyltyrosine or tyrosine using tubulin tyrosine ligase. Samples containing 15 µg of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). A western blot was performed using the OxyBlot™ protein detection oxidation kit according to the manufacturer's instructions. The result of the western blot was visualized by chemiluminescence method (*6*) using SuperSignal West Pico chemiluminescent substrate kit as per the manufacturer's protocol. The results are shown in Figure S1 below.

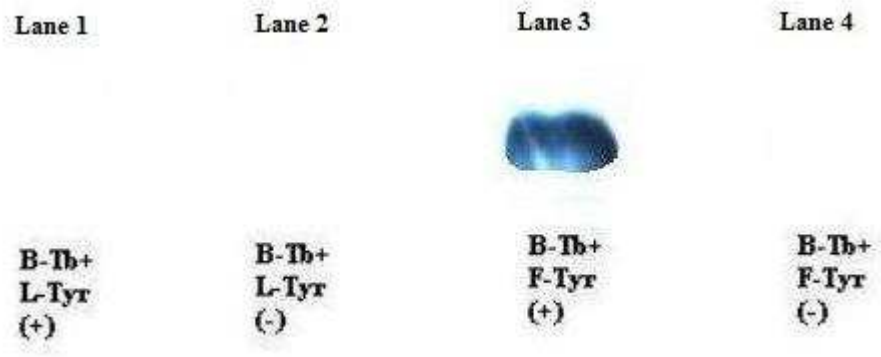


Figure S1: *Bovine brain tubulin labeled* with *L-tyrosine (Lanes 1 and 2) and 3-formyltyrosine (Lanes 3 and 4) was treated with (+) and without (-) DNP hydrazine. A*

western blot using anti-DNPH antibody was performed using OxyBlot™ protein detection oxidation kit.

Subtilisin digestion of tubulin

Limited proteolysis of tubulin using subtilisin is known to selectively cleave the C-terminal peptides from α- and β-tubulin (*7*).

Bovine brain tubulin was labeled with 3-formyltyrosine using TTL as described under Experimental Methods. The tubulin (1 mg/ml) was then treated with subtilisin (1 % w/w) in PME buffer containing 2 mM GTP. The enzyme-tubulin solution was incubated at 25 °C (*7*). Aliquots containing 20 µg of tubulin were collected over a 90 minute period. Each aliquot was quenched with phenyl methyl sulfonyl fluoride (PMSF) to a final concentration of 4 mM and incubated in ice for 30 min. The samples were subjected to SDS PAGE. A western blot using an antibody specific for tyrosinated tubulin (**TUB-1A2**). The results are shown in Figure S2 below.

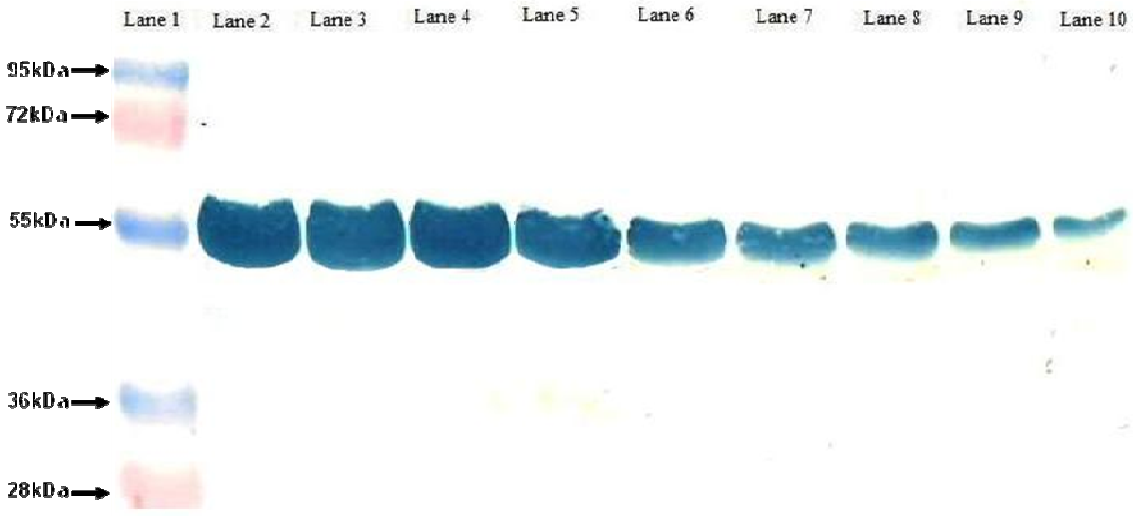
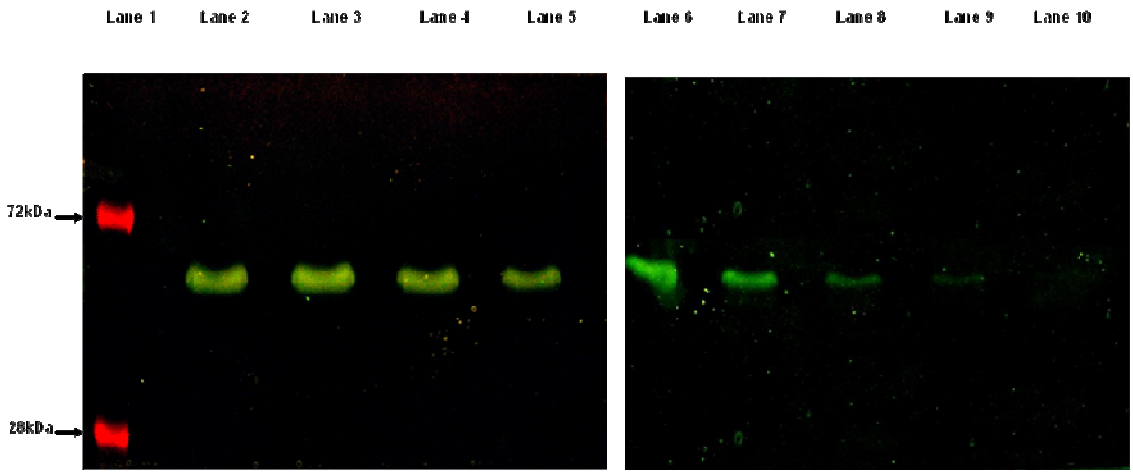


Figure S2*: Bovine brain tubulin incorporated with 3-formyltyrosine was treated with subtilisin in PME buffer (1 % w/w of tubulin). A time course reaction was performed where aliquots (~10 µg protein) and quenched with PMSF in isopropanol and incubated*

for 30 min on ice. All the aliquots were subjected to SDS PAGE. The gel was run for 90 min and then blotted against PVDF membrane for another 90 min. After western blot, the membrane was treated with mouse anti- tyr anti alpha-tubulin as 1^o antibody followed by goat anti-mouse Ig HRP-conjugate as 2^o antibody. The membrane was then treated with TMB (substrate for peroxidase enzyme).
Lane 1 is molecular weight marker. Lane 2 and lane 3 are samples quenched with same volume of isopropanol with and without PMSF. In the following lanes, all the samples were quenched with PMSF in isopropanol. Lane 4 is the aliquot removed immediately after adding subtilisin (t = 0). Lanes 5 to 10 are aliquots removed after 5, 20, 30, 45, 60 and 90 min, respectively.

In a separate experiment, bovine brain tubulin labeled with 3-formyl tyrosine was reacted with coumarin hydrazine as described under Experimental Methods. The fluorescently-labeled tubulin was digested with subtilisin as described above. The samples were subjected to SDS PAGE. The gel was first photographed under long wavelength UV irradiation using a hand held lamp. The same gel was stained with coomassie blue and photographed. The results are shown in Figure S3 below.

Panel A



Panel B

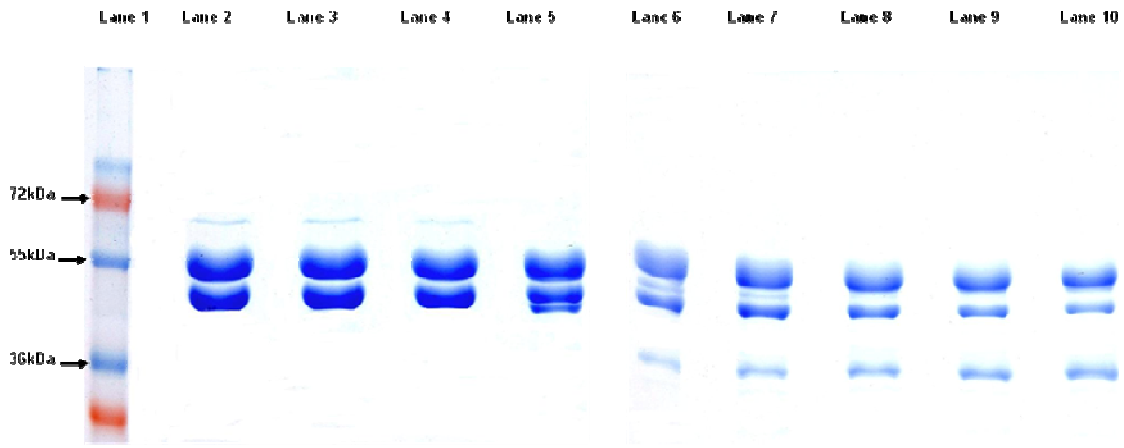


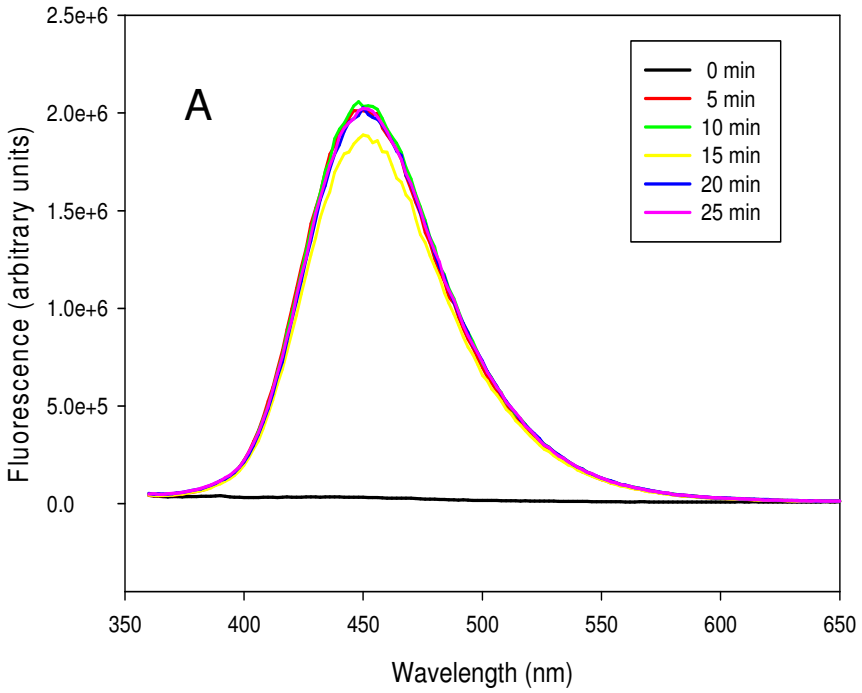
Figure S3*: Fluorescent tubulin (1 mg/ml) was digested with subtilisin in PME buffer (1 % w/w of tubulin). Aliquots (~ 20 µg protein) were removed and quenched with PMSF in isopropanol and incubated for 30 min on ice. All the aliquots were subjected to SDS PAGE. The gel was observed under long wavelength UV lamp (**Panel A**) and then stained with coomassie blue followed by destaining (**Panel B**) and photographed.*

Lane 1 is molecular weight marker. Lane 2 and lane 3 are samples quenched with same volume of isopropanol with and without PMSF. In the following lanes, all the samples were quenched with PMSF in isopropanol. Lane 4 is the aliquot removed immediately after adding subtilisin (t = 0). Lanes 5 to 10 are the aliquots removed after 5, 20, 30, 45, 60 min.

Cellular influx and efflux of coumarin hydrazine

Influx: PC3 (human prostate cancer) cells were grown in 6 well plates to 50 % confluence in RPMI 1640 (Gibco). The medium was removed and fresh medium supplemented with 50 µM coumarin hydrazine (CH) was added and the cells were incubated at 37 °C for 0, 5, 10, 15, 20 and 25 min. The medium was removed and 0.2 ml of 1 % SDS in 10mM Tris (pH 7.4) was added to each well and swirled to cover the whole area. The cells were scraped with a cell scraper and collected to one side of the well. The lysate was removed with a pipette to a microcentrifuge tube, which was then vortexed, boiled for ~ 1 min and sonicated. Fluorescence emission spectra of the cell lysates were measured using a Spex Fluoromax 3 fluorometer at 25 ^{o}C using an excitation

wavelength of 346 nm. Efflux: PC3 cells were grown in 6 well plates to 50 % confluence in RPMI 1640. The medium was removed and fresh medium supplemented with 50 μM CH was added and the cells were incubated at 37 °C for 1 hour. The medium was discarded and the cells were incubated with CH-free medium for 0, 5, 10, 15, 20, 25 and 30 min. Cell lysates were prepared and analyzed for CH fluorescence as described for the influx experiment.



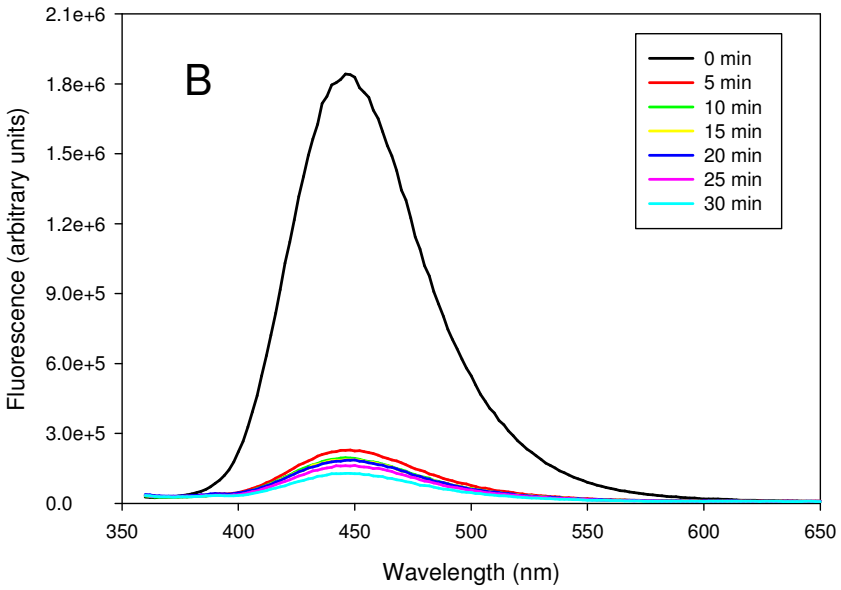


Figure S4. *Influx and efflux of CH is rapid. Panel A. PC3 cells exposed to CH accumulate steady-state levels in less than 5 minutes. Panel B: CH is removed from PC3 cells after a 5 minute wash. The relative amount of CH in PC3 cells was assessed by monitoring CH fluorescence in cell lysates, prepared as described above. The excitation wavelength was 346 nm.*

References:

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