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## Site-Specific Orthogonal Labeling of the Carboxy Terminus of $\alpha$ -Tubulin

Abhijit Banerjee<sup>‡</sup>, Timothy D. Panosian<sup>‡, \*\*</sup>, Kamalika Mukherjee<sup>‡</sup>, Rudravajhala Ravindra<sup>‡</sup>, Susannah Gal<sup>‡</sup>, Dan L. Sackett<sup>§</sup>, and Susan Bane<sup>‡, \*</sup>

<sup>‡</sup>Department of Chemistry, Binghamton University, State University of New York, Binghamton, New York 13902

<sup>‡</sup>Department of Biological Sciences, Binghamton University, Bethesda MD 20892

<sup>§</sup>Laboratory of Integrative and Medical Biophysics, Program in Physical Biology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda MD 20892

### Abstract

A fluorescent probe has been attached to the carboxy terminus of the  $\alpha$ -subunit of  $\alpha, \beta$ -tubulin by an enzymatic reaction followed by a chemical reaction. The unnatural amino acid 3-formyltyrosine is attached to the carboxy terminus of  $\alpha$ -tubulin through the use of the enzyme tubulin tyrosine ligase. The aromatic aldehyde of the unnatural amino acid serves as an orthogonal electrophile that specifically reacts with a fluorophore containing an aromatic hydrazine functional group, which in this case is 7-hydrazino-4-methyl coumarin. Conditions for covalent bond formation between the unnatural amino acid and the fluorophore are mild, allowing fluorescently labeled tubulin to retain its ability to assemble into microtubules. A key feature of the labeling reaction is that it produces a red shift in the fluorophore's absorption and emission maxima, accompanied by an increase in its quantum yield; thus, fluorescently labeled protein can be observed in the presence of unreacted fluorophore. Both the enzymatic and coupling reaction can occur in living cells. The approach presented here should be applicable to a wide variety of *in vitro* systems.

### INTRODUCTION

Microtubules are a fundamental component of the cytoskeleton of eukaryotic cells and are associated with virtually any activity of a cell that involves movement (1). They are part of the mitotic spindle in mammalian cells, and their proper organization is essential for normal cell processes. Although microtubules perform heterogeneous tasks in cells, their basic structure is uniform. The core of the microtubule is entirely composed of tubulin, a 100 kDa heterodimer that assembles to form dynamic cylindrical structures (2).

The carboxy-terminal 15–20 amino acids of each tubulin subunit are the primary locus of sequence heterogeneity in an otherwise highly conserved protein. Tubulin is subject to extensive posttranslational modifications, including acetylation, polyglutamylation, polyglycylation, phosphorylation, tyrosination, and palmitoylation (for recent reviews, see: (3,4)). With the exception of acetylation, all of these posttranslational modifications take

\*Corresponding Author: Phone: 607-777-2927. Fax: 607-777-4478. sbane@binghamton.edu.

\*\*Present address Department of Pharmacology, Vanderbilt University Medical Center, Nashville TN, 37232

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place in the carboxy-terminal peptides of each subunit. Little is known about the structure of these peptides: they are not observable in the electron or X-ray diffraction structures of tubulin (5,6). These peptides contain an abundance of glutamic acid residues and so are highly negatively charged at physiological pH. Molecular modeling supports the earlier hypothesis that the carboxy termini extend into solution perpendicular to the microtubule central axis (7), but there are no experimental data that directly address this question.

A posttranslational event that is unique to tubulin is removal and replacement of the C-terminal tyrosine of  $\alpha$ -tubulin (8). In this process, the genetically encoded tyrosine is cleaved by an unknown carboxypeptidase and replaced by the enzyme tubulin tyrosine ligase (TTL). TTL has been isolated from brain tissue and the human version has been cloned and expressed, but the carboxypeptidase(s) involved in the detyrosination reaction has not yet been identified (9,10). *In vivo*, microtubules containing detyrosinated tubulin (Glu-tubulin) are more stable than those containing tyrosinated tubulin (Tyr-tubulin) (11). Detyrosination does not affect the dynamic or drug-binding properties of purified tubulin (12) and detyrosination of tubulin does not stabilize microtubules *in vitro* (13); thus, the presence or absence of the  $\alpha$ -tubulin carboxy-terminal tyrosine affects the association of non-tubulin proteins with cellular microtubules rather than their intrinsic dynamicity. Although the purpose of the enzymatic cycle is not well understood, it is essential for the life of the cell. There is clear evidence that the tyrosination/detyrosination cycle is critical for neuronal organization and may influence tumorigenesis and tumor invasion. For example, TTL null mice undergo normal embryonic development but die shortly after birth (11). Poor patient prognosis has been correlated with elevated levels of Glu-tubulin in breast and prostate tumors and in neuroblastomas (14–16).

The goal of this research is to develop spectroscopic probes suitable for assessing dynamic properties of the carboxy terminus of tubulin that minimally perturb the peptide structure. Since native mammalian tubulin is very difficult to express in *E. coli* (17), molecular biology techniques will not produce quantities required for *in vitro* experiments. The size of the probe is important, because even a small peptide appended to a carboxy terminus can affect the cellular function of tubulin (18). Our approach is to take advantage of the high target specificity of TTL to attach a modified tyrosine residue to  $\alpha$ -tubulin. The tyrosine derivative possesses a reactive functional group that is orthogonal to the endogenous amino acids. The modified protein can then be reacted with a probe that contains a complementary reactive group. The process is both specific and versatile – that is, a single site on the protein will be covalently labeled, but the nature of the fluorophore (or alternate probe such as a spin label) can be varied. One enzymatic reaction can form the basis for multiple labels.

The chemistry we chose to use is the well known reaction of hydrazone formation. The properties of both the carbonyl and the hydrazine reagents were carefully considered. Purified tubulin is notoriously sensitive to changes in pH and temperature (19), so it is important that the labeling reaction be accomplished as rapidly as possible, near neutral pH and at temperature of 37°C or less.

The strategies we are developing for tubulin labeling form the basis of a broadly applicable approach for site specific labeling of a protein with a wide variety of probes.

## RESULTS AND DISCUSSION

### Tubulin tyrosine ligase: substrate specificity

The only cellular substrate that has been identified for TTL is the C-terminus of  $\alpha$ -tubulin that terminates in the sequence Glu-Glu (8). The enzyme accepts some variation in the structure of the amino acid substrate, although the efficiency of incorporation is usually

substantially lower than that for the normal L-tyrosine substrate. There does not appear to be enough flexibility in the structure of the substrate to attach a fluorescent amino acid such as a dansyl or coumarin derivative using the enzyme (20,21). Aromatic amino acids with minor structural differences such as L-phenylalanine, 3-iodotyrosine, 3-azidotyrosine and 3-fluorotyrosine are weak substrates for TTL and are inhibitors of the enzyme (22–25). The unnatural amino acid 3-nitrotyrosine can also be incorporated into tubulin by the isolated human enzyme and in live cells (26–28).

Inhibition of [ $^3\text{H}$ ]-L-tyrosine incorporation into tubulin by TTL was measured for several aromatic amino acid derivatives to evaluate their potential as substrates for the enzyme (Table 1). Replacing the 4-hydroxyl group with an amine produced no inhibition of TTL at millimolar concentrations; thus, subsequent amino acids tested retained the 4-hydroxyl functionality. The 3-substituted derivatives tested inhibited [ $^3\text{H}$ ]-L-tyrosine incorporation into tubulin at low millimolar concentrations. Two tyrosine derivatives possessing functional groups with orthogonal reactivity were evaluated in this assay. Both 3-azidotyrosine and 3-formyltyrosine inhibited the enzyme to approximately the same extent as 3-nitrotyrosine. Since 3-nitrotyrosine is a substrate for TTL and has been successfully incorporated into  $\alpha$ -tubulin in cultured cells (11), both of these amino acids should be potential chemical labels for tubulin in cells as well as for purified tubulin through TTL.

Orthogonal labeling of tubulin using 3-azidotyrosine should be accessible through 1,3-dipolar reaction with an alkyne-containing label (29). Unless the alkyne is strained, however, the reaction requires copper (I) ion (30), and it is unlikely that tubulin will retain assembly activity after exposure to copper ion (31). We therefore chose to focus on labeling tubulin with the aromatic aldehyde 3-formyltyrosine.

Removal and replacement of the C-terminal tyrosine of  $\alpha$ -tubulin can be assessed by western blot. The antibody **TUB-1A2** is specific for  $\alpha$ -tubulin possessing a C-terminal tyrosine but does not recognize de-tyrosinated tubulin (32). Figure 1 shows that removal of the C-terminal tyrosine of bovine brain  $\alpha$ -tubulin abolishes the antibody binding to the protein, while retyrosination with TTL restores it. Tubulin that has been treated with 3-formyltyrosine in the presence of TTL can be observed in the western blot, indicating that the antibody is also able to recognize the formylated C-terminus. The presence of a carbonyl in  $\alpha$ -tubulin was confirmed reacting the protein with dinitrophenylhydrazine (DNPH) followed by western blot using an anti-DNPH antibody (Supplementary Figure S1).

### Hydrazone formation: Effect of the structure of the nucleophile

Reactions of aldehydes and ketones with hydrazines, hydroxylamines and amines have been extensively studied (33). The rate vs. pH curve is bell shaped; the maximum reaction rate is around the pKa of the hydrazine. Phenyl hydrazide has a pKa of 3.1, four log units away from the desired reaction pH, while phenyl hydrazine has a pKa of 5.3 (34). This feature is significant for bioorthogonal labeling under physiological conditions. Almost all of the commercially available fluorophores for fluorescent labeling of aldehydes and ketones are hydrazides, which are poorly reactive at neutral pH. We hypothesized that a fluorophore possessing a hydrazine functional group would react faster at neutral pH. An aromatic hydrazine coumarin derivative was therefore synthesized. The compound 7-hydrazino-4-methyl coumarin (coumarin hydrazine, Figure 2) was allowed to react with 3-formyltyrosine and the kinetics of hydrazone formation were followed by absorption difference spectroscopy. Hydrazone formation is complete within 120 min under these conditions: the second order rate constant is  $53 \text{ M}^{-1}\text{min}^{-1}$  (Figure 3, panel A). Under the same conditions, reaction of the commercially available coumarin hydrazide (Figure 2) was barely detectable after 4 h. The second order rate constant for this reaction was estimated to be less than  $2 \text{ M}^{-1}\text{min}^{-1}$ .

It should be possible to increase the reaction rate of hydrazone formation using aniline as a catalyst, as shown by Dirksen and Dawson (35). In the absence of such a catalyst, though, it is clear that an aromatic hydrazine and not a hydrazide is better suited for the tubulin labeling reaction. There are other reasons why an aromatic hydrazine is a good choice for the nucleophile. Hydrazones formed from aromatic hydrazines are more stable than those formed from aliphatic hydrazines, which are in turn more stable than those formed from hydrazides (34).

Another important feature of a hydrazine reactive group is the possibility that hydrazone formation may alter the electronic properties of the fluorophore. For example, if the hydrazino functional group is directly bonded to the chromophore, formation of the hydrazone may provide conjugation between the aromatic aldehyde and the fluorescent hydrazine. Covalent bond formation may shift the absorption and emission maxima of some fluorophores to longer wavelengths than the corresponding hydrazine (36). In addition, fluorophores possessing aromatic amines are frequently weakly fluorescent, often due to intramolecular photoelectron transfer involving the amine lone pair. Structural modifications that delocalize the electron density on the amine nitrogen, such as amide bond formation, can greatly increase the quantum yield of the fluorophore (37). It is therefore possible that feebly fluorescent hydrazines may become highly fluorescent species upon hydrazone formation; that is, hydrazone formation may “turn on” the fluorophore (38). Thus, covalently bound fluorophore may be readily detectable in the presence of non-covalently bound fluorophore.

To test this hypothesis, we measured absorption and fluorescence spectra of coumarin hydrazine and the salicylaldehyde hydrazone of coumarin hydrazine, which serves as a model compound for the protein-bound hydrazone. Hydrazone formation causes a shift in both the absorption and emission maxima of coumarin hydrazine and a significant increase in quantum yield (Table 2). Thus, as a result of the difference in the spectroscopic window and the quantum yields of coumarin hydrazine and its salicylaldehyde hydrazone, fluorescence from unreacted coumarin hydrazine can be averted.

### Orthogonal labeling of native tubulin

Figure 3, panel B shows that coumarin hydrazine reacts with 3-formyltyrosinated tubulin to form a fluorescent product. Its emission maximum is red shifted relative to that of unreacted coumarin hydrazine, indicating that a hydrazone bond is formed. To assess if a covalent bond is formed with just one subunit, tubulin was detyrosinated with carboxypeptidase A and then retyrosinated with TTL using either L-tyrosine or 3-formyltyrosine. Each protein sample was incubated with coumarin hydrazine. SDS-PAGE of the two samples (Figure 4) reveals a single fluorescent band for 3-formyltyrosine-treated tubulin, which corresponds to  $\alpha$ -tubulin. No fluorescence is observed in the control samples. Limited proteolysis with subtilisin caused a time-dependent decrease in the fluorescence intensity of the  $\alpha$ -tubulin band (Supplementary Figure S3), which is consistent with the idea that the fluorophore is attached to the C-terminus of  $\alpha$ -tubulin (39). The fluorescently-labeled tubulin retains its ability to polymerize, which is illustrated for bovine brain tubulin in Figure 5.

### Hydrazone Formation in Cells

Preliminary experiments to evaluate the suitability of the fluorophore for live cell imaging have been performed using PC3 cells. The fluorophore is quickly taken up by the cells and can be rapidly removed by washing (Supplementary Figure S4). Figure 6 shows cells that have been grown in tyrosine depleted medium supplemented with either 50  $\mu$ M L-tyrosine or 50  $\mu$ M 3-formyltyrosine and then have been treated with 20  $\mu$ M coumarin hydrazine. Images were obtained shortly after addition of the probe (15–20 min), which was not

removed from the medium. Cells grown in 3-formyltyrosine are significantly brighter than those grown in L-tyrosine. Thus, the reaction between the unnatural amino acid and the hydrazine can occur in and can be observed in an intracellular environment.

### Tubulin Labeling in Cells

This two step labeling process can be used to fluorescently tag  $\alpha$ -tubulin in live cells. An initial assessment has been performed using CHO cells. Cells grown in tyrosine depleted medium were treated overnight with equal concentrations of either L-tyrosine or 3-formyltyrosine followed by a 2 h incubation with coumarin hydrazine. Proteins of the cell lysate were separated using SDS-PAGE. A single fluorescent protein was observed in the lysate, which was identified by western blot to be  $\alpha$ -tubulin (Figure 7).

## CONCLUSION

We have successfully used an *in vitro* system for adding a 3-formyltyrosine residue to just the C-terminus of  $\alpha$ -tubulin, which is highly specific both in purified protein and in cultured cells. The modified tyrosine, which remains a substrate for TTL, possesses a chemically reactive group that is orthogonal to the amino acid residues of the protein. This unnatural amino acid is capable of reacting with appropriately functionalized probes to form a covalent bond solely between the modified amino acid and the label. Covalent bond formation between the unnatural amino acid and the fluorophore can take place at moderate temperatures at neutral pH, which are conditions amenable to covalent bond formation in live cells.

Furthermore, the fluorescent probe prepared for this work provides additional proof in principle that a hydrazine bonded directly to a fluorophore  $\pi$ -system may alter the optical properties of a fluorophore upon hydrazone formation (36). The fluorophore is weakly fluorescent in a polar environment; covalent bond formation causes it to “turn on”. Thus, owing to the shift in absorption and emission maxima in conjunction with an increase in the quantum yield, one may observe only those proteins that possess the appropriate reactive group in the presence of unreacted probe.

Aspects of this project should be applicable to other systems. For example, aromatic hydrazine-containing fluorophores that “turn on” when a hydrazone bond is formed may be useful for labeling other proteins containing aldehydes, such as cell surface glycoproteins. We are preparing other fluorophores with similar reactive and fluorescent properties for this purpose (36). Ideally, the observation window for the fluorescently-labeled protein can be changed simply by selecting a different reactive fluorophore for the experiment. This two step labeling system should be broadly applicable to site-specific labeling of proteins using unnatural amino acid mutagenesis methodology (40,41). Methyl ketone derivatives of phenylalanine have already been developed for this purpose. Hydrazone formation with an *o*-hydroxy benzaldehyde moiety occurs more rapidly than with an unsubstituted aromatic ketone (42,43). The 3-formyltyrosine could be considered as an alternative electrophile if a more reactive group is required for a particular application. We have preliminary results to support this idea, which will be presented in due course.

## METHODS

### Synthesis

The unnatural amino acid 3-formyltyrosine was synthesized from BOC-L-tyrosine by the Reimer-Tiemann reaction followed by deprotection with trifluoroacetic acid in dichloromethane. The fluorophore 7-hydrazino-4-methyl coumarin was synthesized from 7-amino-4-methyl coumarin by treatment with nitrous acid followed by  $\text{SnCl}_2$ . The



salicylaldehyde hydrazone of coumarin hydrazine was prepared in ethanol with trifluoroacetic acid catalysis. Procedures and spectroscopic data are detailed in Supporting Information.

### Tubulin tyrosine ligase

An initial supply of TTL was a gift of Prof. Dr. Jürgen Wehland and Dr. Christian Erck (Gesellschaft für Biotechnologische Forschung, Germany) and was stored at  $-80^{\circ}\text{C}$ . Human TTL was prepared using expression vector pReceiver 05x (Genecopoeia, Maryland), transformed into the *Escherichia coli* expression strain BL21. Additional experimental details are in Supporting Information. Equivalent results were obtained with TTL from both sources.

### Tubulin preparation

Tubulin was isolated from bovine brain by two cycles of assembly/disassembly followed by phosphocellulose chromatography and was stored in liquid nitrogen until use (44). Tubulin was detyrosinated by combining  $30\ \mu\text{M}$  tubulin in PME buffer ( $0.1\ \text{M}$  PIPES,  $1\ \text{mM}$   $\text{MgSO}_4$ ,  $2\ \text{mM}$  EGTA,  $\text{pH } 6.90$ ) with  $0.1\ \text{mg ml}^{-1}$  carboxypeptidase A (Sigma) and allowed to react  $30\ \text{min}$  at  $37^{\circ}\text{C}$ . The reaction was stopped by the addition of DTT to  $20\ \text{mM}$ . The cleaved tyrosine was removed and buffer was exchanged by rapid gel filtration using Sephadex G-50 in PME buffer. Removal of the C-terminal tyrosine was confirmed by western blot with the tyrosinated tubulin specific antibody **TUB-1A2** (Sigma).

Detyrosinated tubulin was drop frozen in liquid nitrogen and stored in liquid nitrogen until use. To retyrosinate tubulin with either L-tyrosine or 3-formyltyrosine, detyrosinated tubulin was thawed and equilibrated in TTL buffer using gel filtration chromatography (TTL buffer =  $25\ \text{mM}$  MES,  $150\ \text{mM}$  KCl,  $27\ \mu\text{M}$   $\text{MgCl}_2$ ,  $2.5\ \text{mM}$  ATP,  $1\ \text{mM}$  DTT and  $1.5\%$  glycerol  $\text{pH } 6.8$ ). A final concentration of  $10\ \mu\text{M}$  tubulin was combined with  $1\ \text{mM}$  ligand (L-tyrosine or 3-formyltyrosine) and  $0.1\ \text{mg ml}^{-1}$  TTL and allowed to react for  $30\ \text{min}$  at  $37^{\circ}\text{C}$ . Excess ligand was removed by rapid gel filtration into PME buffer. Control experiments in which TTL was excluded from the sample were also performed. The presence of a carbonyl in just the tubulin sample treated with TTL and 3-formyltyrosine was confirmed using the OxyBlot™ Protein Oxidation Detection Kit (Chemicon International).

### [ $^3\text{H}$ ]-Tyr incorporation assay

TTL was thawed and diluted to  $1\ \text{mg ml}^{-1}$  immediately before use. Detyrosinated tubulin ( $10\ \mu\text{M}$  tubulin) in TTL buffer was combined with  $0.007\ \mu\text{Ci } ^3\text{H-Tyr}$ ,  $0.1\ \mu\text{g } ^3\text{H-Tyr}$  and various concentrations of inhibitor. The mixture was allowed to react for  $30\ \text{min}$  at  $37^{\circ}\text{C}$ . A  $35\ \mu\text{l}$  aliquot of the sample was placed onto DEAE chromatography paper (Whatman) and allowed to dry for  $30\ \text{min}$ . The protein was fixed using  $200\ \mu\text{l}$   $10\%$  acetic acid and allowed to dry for  $10\ \text{min}$ . Excess [ $^3\text{H}$ -Tyr] was removed by 2 washes with  $3.5\ \text{ml}$  of  $100\%$  ethanol. The paper was allowed to dry for  $30\ \text{min}$  and then was combined with  $10\ \text{ml}$  Scintiverse (BD) scintillation fluid. The amount of [ $^3\text{H}$ ]-tyrosine on each filter was determined using a  $\beta$ -counter.

### Kinetic measurements

Kinetics of hydrazone formation were measured using absorption difference spectroscopy. Dual chamber cells were loaded with identical volumes of 3-formyltyrosine ( $50\ \mu\text{M}$ ) and the selected nucleophile (coumarin hydrazine or coumarin hydrazide,  $500\ \mu\text{M}$ ). The cell was placed in a thermostatted cell holder in an HP 8453 UV-Vis spectrophotometer and equilibrated to  $25^{\circ}\text{C}$ . A baseline was established by blanking the instrument with the dual chamber cell. The solutions were mixed by rapid inversion of the cell (which doubles the path and halves the concentration of each component). Absorption difference spectra were

collected as a function of time. The change in absorbance for one wavelength was selected from the spectral data, and these values were plotted as a function of time. The data were analyzed as a single pseudo-first order reaction and were fit to the appropriate equation using SigmaPlot 10.0.

### Fluorescent labeling of tubulin with coumarin hydrazine

Coumarin hydrazine was freshly prepared by dissolving the solid material in PME buffer, which was then centrifuged to remove undissolved particles. The concentration of coumarin hydrazine was determined by absorption spectroscopy (using  $\epsilon_{346\text{ nm}} = 1.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  in PME buffer). Tubulin used in these experiments was detyrosinated using CPA and then retyrosinated with tyrosine (control) or 3-formyltyrosine using TTL prior to use as described above. Each tubulin sample was incubated with coumarin hydrazine in at least 1:10 molar ratio (tubulin:coumarin hydrazine) at room temperature for 120 min. Unreacted fluorophore was removed by rapid gel filtration. Labeled tubulin was visualized with a hand held UV lamp after SDS-PAGE. An additional control experiment was performed in which TTL was excluded from the procedure. The maximum fluorescence intensity of these samples was approximately the same as the tyrosine control (Figure 3, panel B, solid curve).

### Fluorescence of coumarin hydrazine in live cells grown in the presence or absence of 3-formyltyrosine

PC3 cells were grown in 4 chambered cover glass to 50% confluence in Ham's F-10 medium (Sigma Aldrich). Medium was discarded and the cells were incubated with fresh medium supplemented with 50  $\mu\text{M}$  3-formyl tyrosine or L-tyrosine at 37 °C for 24 h. The cells were washed with fresh medium 3 times. Fresh medium supplemented with 20  $\mu\text{M}$  coumarin hydrazine was added. After about 15 min cells were observed under Plan-Neofluar 40x/1.3 Oil DIC (Optical zoom: 2.0) using Zeiss LSM 510 META laser scanning confocal microscope. The cells were imaged using excitation wavelength of 405 nm (violet laser diode). The emission was monitored using band pass (BP) 420–480. Microscope instrumental gain settings were the same for cell incubated with tyrosine or 3-formyltyrosine.

### Fluorescent labeling of tubulin in cells

Chinese hamster ovary (CHO) cells were grown in Ham's F-10 (tyrosine depleted) medium in six well plates. Semiconfluent cells were treated with 50  $\mu\text{M}$  tyrosine or 3-formyltyrosine in medium and incubated at 37 °C overnight. The medium was discarded and the cells were treated with 100  $\mu\text{M}$  of coumarin hydrazine in fresh medium and incubated at 37 °C for 2 h. The medium was removed and the cells were washed once with PBS. Lysis buffer (0.2 ml per well for a 6 well plate) 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 and 1  $\mu\text{l ml}^{-1}$  of protease inhibitor cocktail was added. After shearing the extract using a 26.5 gauge needle, the samples were vortexed and then boiled for ~1 min. The extracts were separated using SDS-PAGE. Separate gels were stained for protein, visualized using a hand held UV lamp or were analyzed by western blot using a polyclonal anti- $\alpha$ -tubulin antibody

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.



## Abbreviations

<b>ATP</b>	Adenosine 5'-triphosphate
<b>DEAE</b>	diethylaminoethyl cellulose
<b>DMSO</b>	dimethyl sulfoxide
<b>EGTA</b>	ethylene glycol-bis(2-aminoethylether)- <i>N,N,N',N'</i> -tetraacetic acid
<b>G-50</b>	Sephadex G-50
<b>GTP</b>	guanosine 5'-triphosphate
<b>MAPs</b>	microtubule associated proteins
<b>MgSO<sub>4</sub></b>	magnesium sulfate
<b>PIPES</b>	Piperazine-1,4-bis(2-ethanesulfonic acid), PME, 0.1 M PIPES, 1 mM MgSO <sub>4</sub> , and 2 mM EGTA, pH 6.90 at 25 °C

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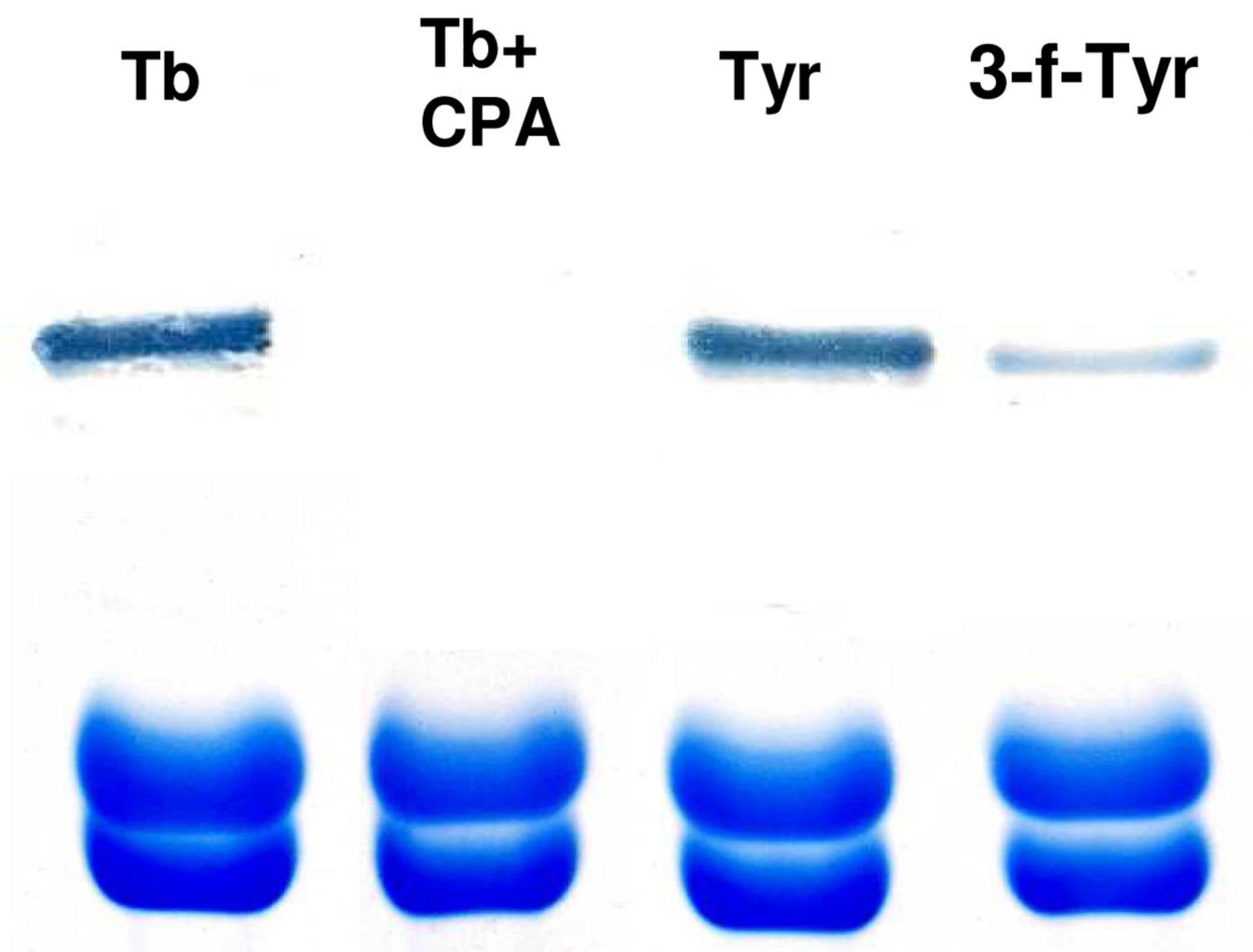
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## REFERENCES

1. Avila, J. Microtubule Proteins. Boca Raton, FL: CRC Press; 1990.
2. Jordan MA, Wilson L. Microtubules and actin filaments: dynamic targets for cancer chemotherapy. *Curr. Opin. Cell Biol.* 1998; 10:123–130. [PubMed: 9484604]
3. Hammond JW, Cai DW, Verhey KJ. Tubulin modifications and their cellular functions. *Curr. Opin. Cell Biol.* 2008; 20:71–76. [PubMed: 18226514]
4. Westermann S, Weber K. Post-translational modifications regulate microtubule function. *Nature Rev. Cell Mol. Biol.* 2003; 4:938–947.
5. Nogales E, Wolf SG, Downing KH. Structure of the alpha beta tubulin dimer by electron crystallography. *Nature.* 1998; 391:199–203. [PubMed: 9428769]
6. Gigant B, Curmi PA, Martin-Barbey C, Charbaut E, Lachkar S, Lebeau L, Siavoshian S, Sobel A, Knossow M. The 4 Å X-ray structure of a tubulin:stathmin-like domain complex. *Cell.* 2000; 102:809–816. [PubMed: 11030624]
7. Priel A, Tuszyński JA, Woolf NJ. Transitions in microtubule C-termini conformations as a possible dendritic signaling phenomenon. *Eur. Biophys. J.* 2005; 35:40–52. [PubMed: 16184388]
8. Erck C, Frank R, Wehland J. Tubulin-tyrosine ligase, a long-lasting enigma. *Neurochem. Res.* 2000; 25:5–10. [PubMed: 10685598]
9. Wehland J, Schroder HC, Weber K. Isolation and purification of tubulin tyrosine ligase. *Methods Enzymol.* 1986; 134:170–179. [PubMed: 3821560]
10. Ersfeld K, Wehland J, Plessmann U, Dodemont H, Gerke V, Weber K. Characterization of the tubulin tyrosine ligase. *J. Cell Biol.* 1993; 120:725–732. [PubMed: 8093886]
11. Erck C, Peris L, Andrieux A, Meissirel C, Gruber AD, Vernet M, Schweitzer A, Saoudi Y, Pointu H, Bosc C, Salin PA, Job D, Wehland J. A vital role of tubulin-tyrosine-ligase for neuronal organization. *Proc. Natl. Acad. Sci. U. S. A.* 2005; 102:7853–7858. [PubMed: 15899979]
12. Skoufias DA, Wilson L. Assembly and colchicine binding characteristics of tubulin with maximally tyrosinated and detyrosinated alpha-tubulins. *Arch. Biochem. Biophys.* 1998; 351:115–122. [PubMed: 9500839]

13. Webster DR, Wehland J, Weber K, Borisy GG. Detyrosination of alpha-tubulin does not stabilize microtubules in vivo. *J. Cell Biol.* 1990; 111:113–122. [PubMed: 1973168]
14. Mialhe A, Lafanechere L, Treilleux I, Peloux N, Dumontet C, Bremond A, Panh MH, Payan R, Wehland J, Margolis RL, Job D. Tubulin detyrosination is a frequent occurrence in breast cancers of poor prognosis. *Cancer Res.* 2001; 61:5024–5027. [PubMed: 11431336]
15. Kato C, Miyazaki K, Nakagawa A, Ohira M, Nakamura Y, Ozaki T, Imai T, Nakagawara A. Low expression of human tubulin tyrosine ligase and suppressed tubulin tyrosination/detyrosination cycle are associated with impaired neuronal differentiation in neuroblastomas with poor prognosis. *Int. J. Cancer.* 2004; 112:365–375. [PubMed: 15382060]
16. Soucek K, Kamaid A, Phung AD, Kubala L, Bulinski JC, Harper RW, Eiserich JP. Normal and prostate cancer cells display distinct molecular profiles of alpha-tubulin posttranslational modifications. *Prostate.* 2006; 66:954–965. [PubMed: 16541425]
17. Shah C, Xu CZQ, Vickers J, Williams R. Properties of microtubules assembled from mammalian tubulin synthesized in *Escherichia coli*. *Biochemistry.* 2001; 40:4844–4852. [PubMed: 11294652]
18. Andresen M, Schmitz-Salue R, Jakobs S. Short tetracysteine tags to beta-tubulin demonstrate the significance of small labels for live cell imaging. *Mol. Biol. Cell.* 2004; 15:5616–5622. [PubMed: 15469986]
19. Prasad ARS, Luduena RF, Horowitz PM. Bis(8-Anilino-naphthalene-1-sulfonate) as a probe for tubulin decay. *Biochemistry.* 1986; 25:739–742. [PubMed: 3955027]
20. Lee HS, Guo JT, Lemke EA, Dimla RD, Schultz PG. Genetic Incorporation of a small, environmentally sensitive, fluorescent probe into proteins in *Saccharomyces cerevisiae*. *J. Am. Chem. Soc.* 2009; 131:12921–12923. [PubMed: 19702307]
21. Katritzky AR, Narindoshvili T. Fluorescent amino acids: advances in protein-extrinsic fluorophores. *Org. Biomol. Chem.* 2009; 7:627–634. [PubMed: 19194572]
22. Arce CA, Rodriguez JA, Barra HS, Caputo R. Incorporation of L-tyrosine, L-phenylalanine and L-3,4-dihydroxyphenylalanine as single units into rat brain tubulin. *Eur. J. Biochem.* 1975; 59:145–149. [PubMed: 1204603]
23. Monasterio O, Nova E, Lopezbrauet A, Lagos R. Tubulin-tyrosine ligase catalyzes covalent binding of 3-fluoro-tyrosine to tubulin - Kinetic and [F-19]NMR Studies. *FEBS Lett.* 1995; 374:165–168. [PubMed: 7589526]
24. Kalisz HM, Erck C, Plessmann U, Wehland J. Incorporation of nitrotyrosine into alpha-tubulin by recombinant mammalian tubulin-tyrosine ligase. *Biochem. Biophys. Acta.* 2000; 1481:131–138. [PubMed: 11004583]
25. Coudijzer K, Joniau M. 3-Azido-L-tyrosine as a photoinhibitor of tubulin - tyrosine ligase. Role of thiol-groups. *FEBS Lett.* 1990; 268:95–98. [PubMed: 2384179]
26. Bisig CG, Purro SA, Contin MA, Barra HS, Arce CA. Incorporation of 3-nitrotyrosine into the C-terminus of  $\alpha$ -tubulin is reversible and not detrimental to dividing cells. *Eur. J. Biochem.* 2002; 269:5037–5045. [PubMed: 12383263]
27. Mihm MJ, Schanbacher BL, Wallace BL, Wallace LJ, Uretsky NJ, Bauer JA. Free 3-nitrotyrosine causes striatal neurodegeneration in vivo. *J. Neurosci.* 2001; 21:1–5. RC149.
28. Eiserich JP, Estevez AG, Bamberg TV, Ye YZ, Chumley PH, Beckman JS, Freeman BA. Microtubule dysfunction by posttranslational nitrotyrosination of alpha-tubulin: A nitric oxide-dependent mechanism of cellular injury. *Proc. Natl. Acad. Sci. U. S. A.* 1999; 96:6365–6370. [PubMed: 10339593]
29. Moses JE, Moorhouse AD. The growing applications of click chemistry. *Chem. Soc. Rev.* 2007; 36:1249–1262. [PubMed: 17619685]
30. Jean-François L. Copper-free azide-alkyne cycloadditions: New insights and perspectives. *Angew. Chem.* 2008; 47:2182–2184. [PubMed: 18264961]
31. Liliom K, Wagner G, Pacz A, Cascante M, Kovacs J, Ovadi J. Organization-dependent effects of toxic bivalent ions - Microtubule assembly and glycolysis. *Eur. J. Biochem.* 2000; 267:4731–4739. [PubMed: 10903506]
32. Rudiger M, Weber K. Characterization of the posttranslational modifications in tubulin from the marginal band of avian erythrocytes. *Eur. J. Biochem.* 1993; 218:107–116. [PubMed: 8243458]

33. Jencks, WP. Mechanism and Catalysis of Simple Carbonyl Group Reactions. In: Cohen, SG.; Streitwieser, A., Jr; Taft, RW., editors. *Prog. Phys. Org. Chem. Vol. Volume 2*. New York: John Wiley & Sons, Inc; 1964. p. 63-128.
34. Smith, PAS. *Derivatives of Hydrazine and Other Hydronitrogens Having N-N Bonds*. Reading, Massachusetts: The Benjamin/Cummings Publishing Company; 1983.
35. Dirksen A, Dawson PE. Rapid oxime and hydrazone ligations with aromatic aldehydes for biomolecular labeling. *Bioconjug. Chem.* 2008; 19:2543–2548. [PubMed: 19053314]
36. Dilek O, Bane SL. Synthesis of boron dipyrromethene fluorescent probes for bioorthogonal labeling. *Tetrahedron Lett.* 2008; 49:1413–1416.
37. Valeur, B. *Molecular Fluorescence Principles and Applications*. Weinheim: Wiley-VCH; 2002.
38. Vogel M, Buldt A, Karst U. Hydrazine reagents as derivatizing agents in environmental analysis - a critical review. *Fresenius J. Anal. Chem.* 2000; 366:781–791. [PubMed: 11227409]
39. Sackett, DL. Structure and Function in the Tubulin Dimer and the Role of the Acidic Carboxy Terminus. In: Biswas, BB.; Roy, S., editors. *Proteins: Structure, Function and Engineering*. New York: Plenum Press; 1995. p. 255-302.
40. Xie JM, Schultz PG. Innovation: A chemical toolkit for proteins -an expanded genetic code. *Nature Rev. Cell Mol. Biol.* 2006; 7:775–782.
41. Liu WS, Brock A, Chen S, Chen SB, Schultz PG. Genetic incorporation of unnatural amino acids into proteins in mammalian cells. *Nature Methods.* 2007; 4:239–244. [PubMed: 17322890]
42. Do Amaral L, Bastos MP. Kinetics and mechanism for benzaldehyde phenylhydrazone formation. *J. Org. Chem.* 1971; 36:3412–3417.
43. Alves KB, Bastos MP, Do Amaral L. Mechanism and catalysis for o-hydroxyacetophenone phenylhydrazone formation. *J. Org. Chem.* 1978; 43:4032–4038.
44. Williams RC, Lee JC. Purification of tubulin from brain. *Methods Enzymol.* 1982; 85:376–385. [PubMed: 7121276]



**FIGURE 1. 3-Formyltyrosine is incorporated into  $\alpha$ -tubulin**

Carboxypeptidase A (CPA) treated tubulin was incubated with tubulin tyrosine ligase in the presence of either L-tyrosine or 3-formyltyrosine as described under Experimental Procedures and then subjected to SDS-PAGE. Top: Western blot with tyrosinated  $\alpha$ -tubulin antibody (Sigma-Aldrich TUB-1A2). Bottom: Coomassie stain. Lane 1: Tubulin. Lane 2: Tubulin treated with CPA. Lane 3: CPA-treated tubulin retyrosinated with tyrosine. Lane 4: CPA-treated tubulin retyrosinated with 3-formyltyrosine. The mass of protein loaded each well was 0.66  $\mu$ g for the western blot and 10  $\mu$ g for the protein stain.

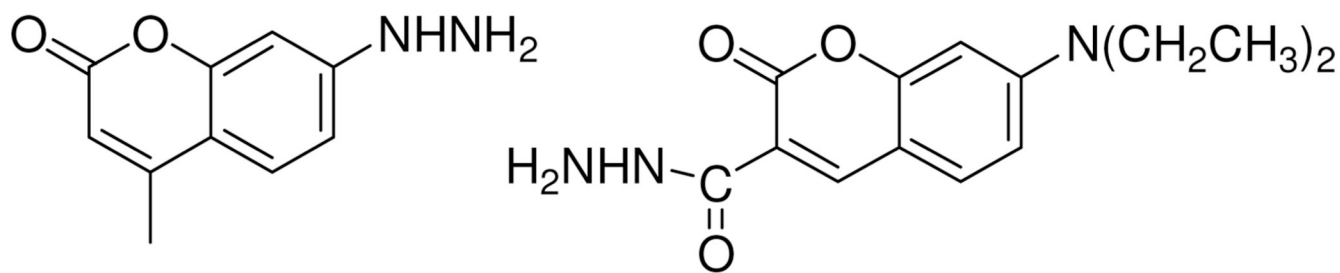
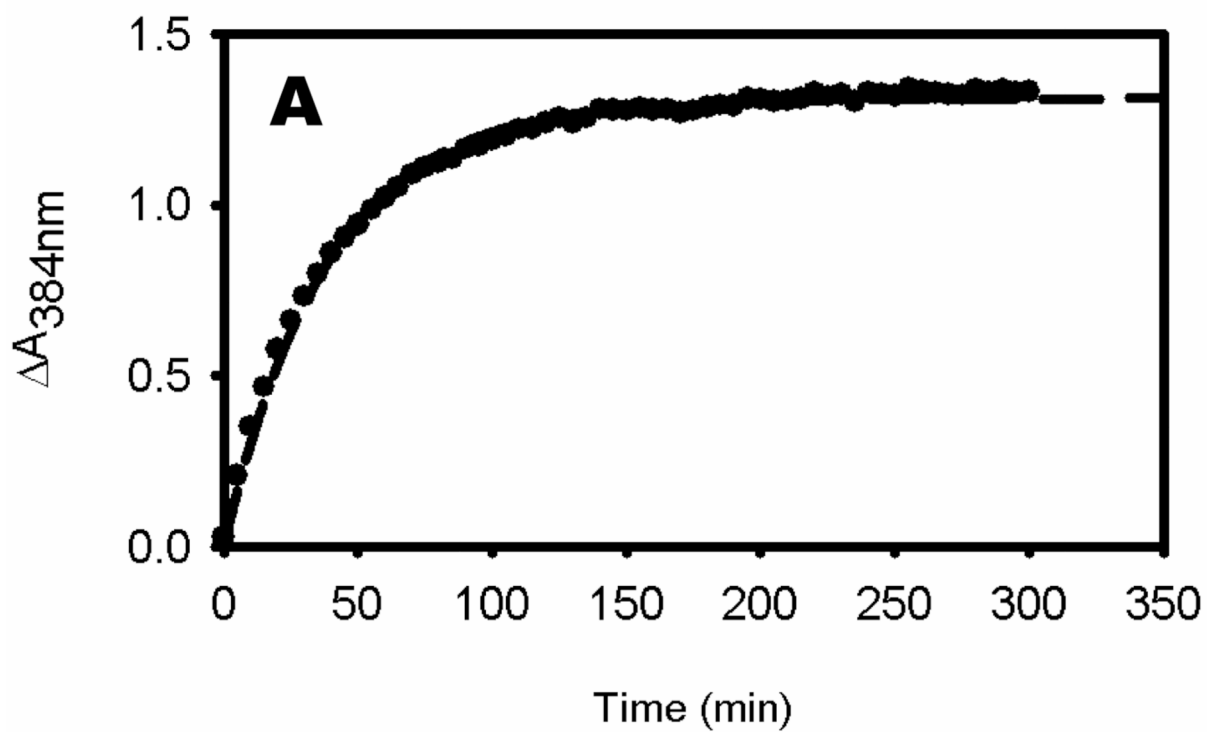
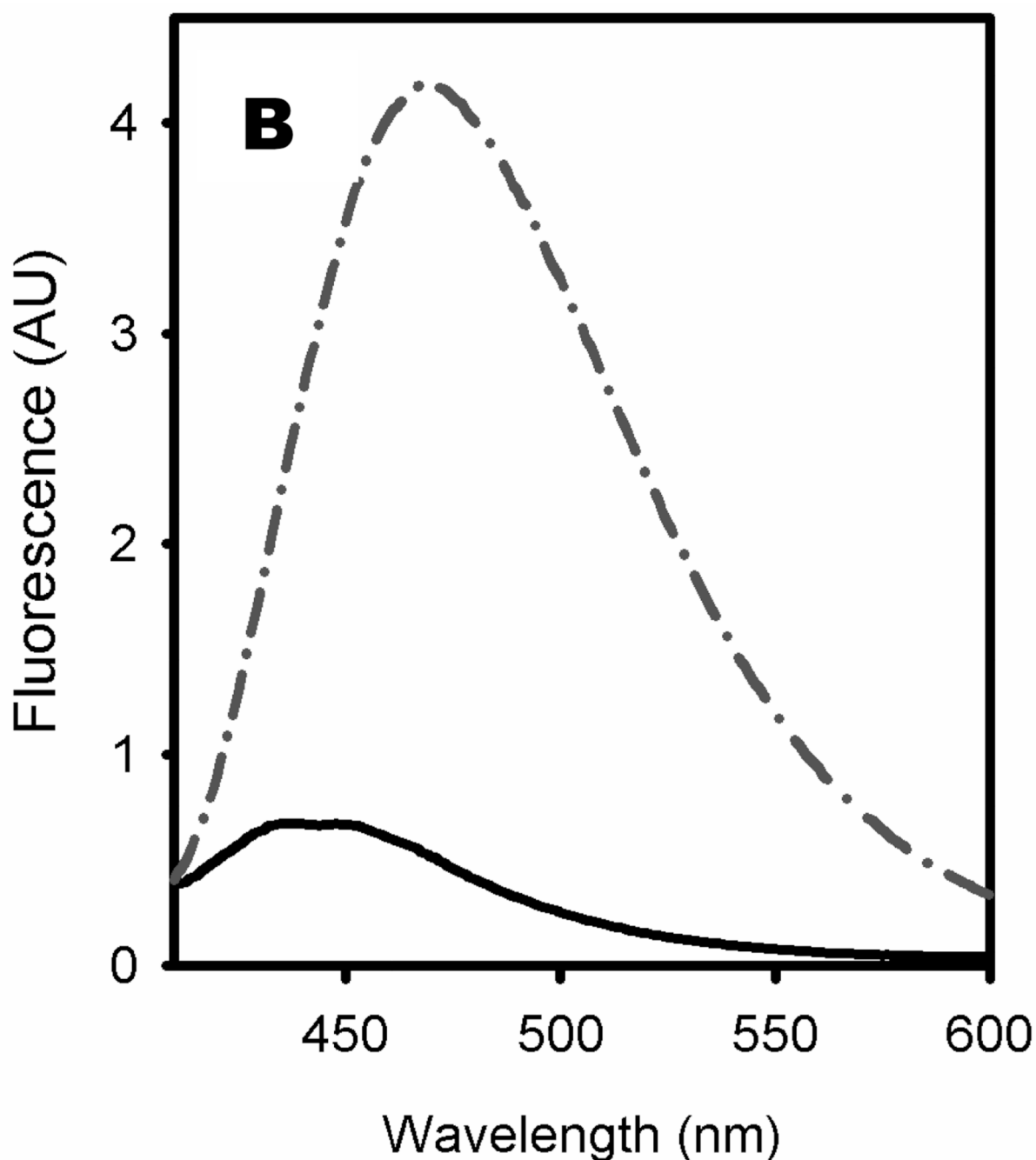


FIGURE 2. Structures of coumarin hydrazine and coumarin hydrazide

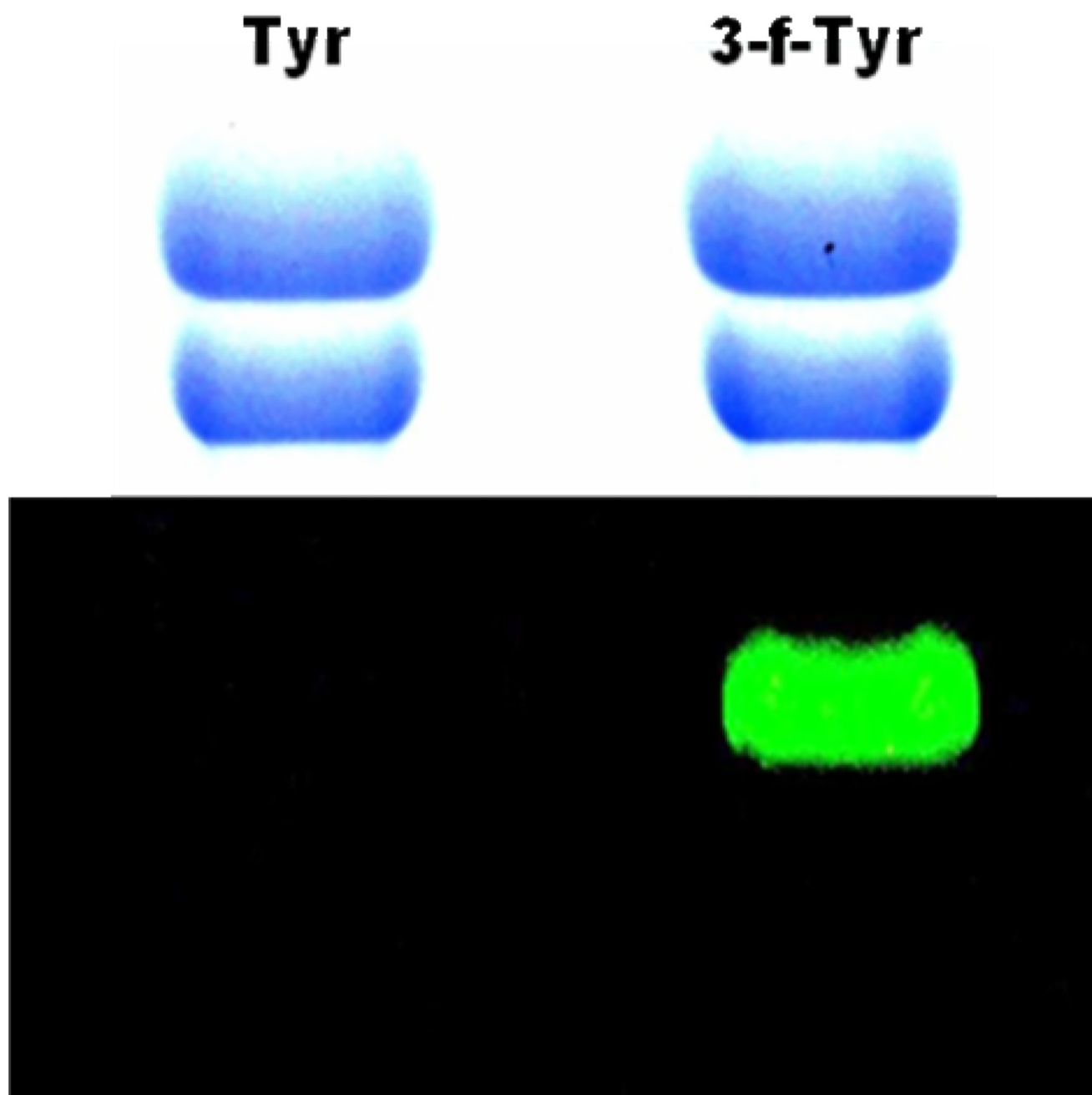




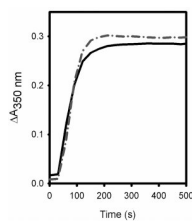


**FIGURE 3.**

**A. Kinetics of hydrazone formation between coumarin hydrazine and 3-formyltyrosine.** Coumarin hydrazine (25  $\mu\text{M}$ ) was mixed with 3-formyltyrosine (250  $\mu\text{M}$ ) in PME buffer (pH 6.9) at 25°C. The absorption difference spectrum was collected at 5 min intervals and the change in absorption at 384 nm was plotted. Solid line: Fit of data to a single exponential. **B. Emission spectrum of fluorescently labeled tubulin.** CPA-treated tubulin was retyrosinated with L-tyrosine (solid curve) or 3-formyltyrosine (dot-dash curve) followed by treatment of coumarin hydrazine as described under Experimental Procedures. Each sample contained 2  $\mu\text{M}$  tubulin. The excitation wavelength was 392 nm.

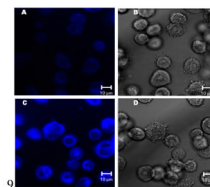


**FIGURE 4. Coumarin hydrazine covalently binds to 3-formyltyrosinated  $\alpha$ -tubulin**  
CPA-treated tubulin, tyrosinated using TTL and 3-formyltyrosine (left) or L-tyrosine (right), at a final concentration of 30  $\mu$ M was treated with 100  $\mu$ M of coumarin hydrazine in PME buffer prior to SDS-PAGE. The upper band in the gel is  $\alpha$ -tubulin and the lower band is  $\beta$ -tubulin. Top: Coomassie stain. Bottom: Fluorescence under long wavelength UV lamp.



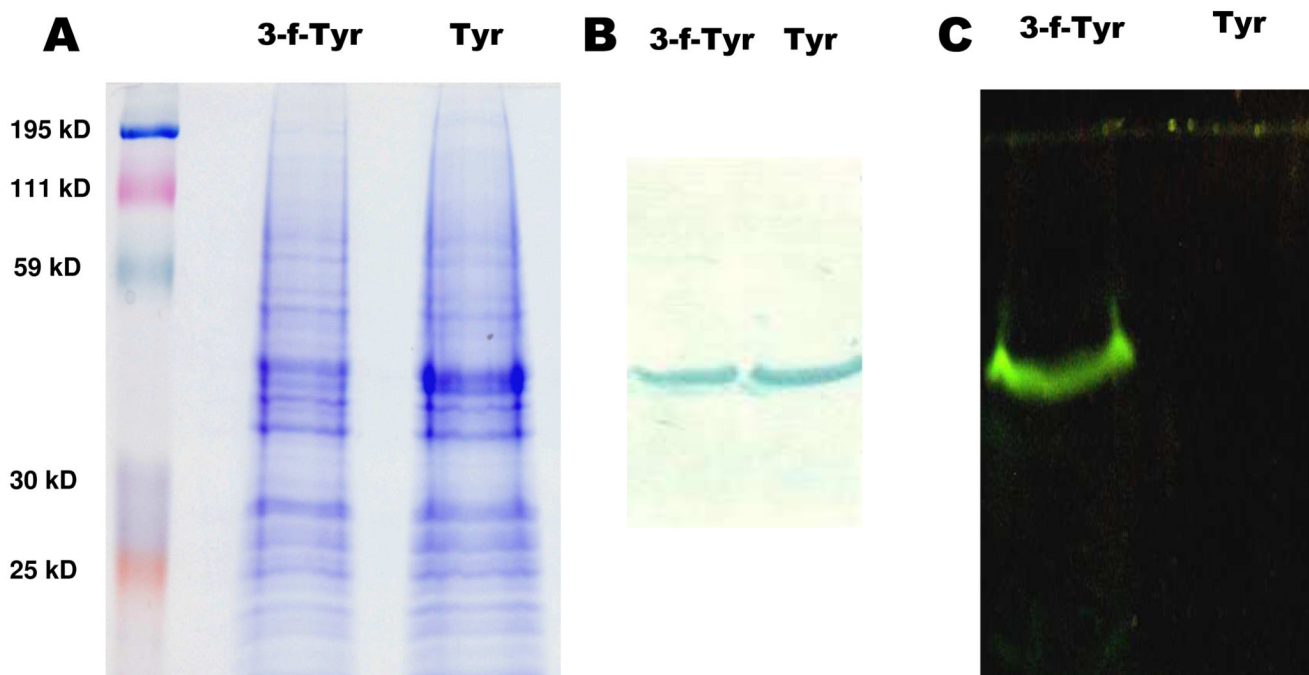
**FIGURE 5. Fluorescently-labeled tubulin retains assembly activity**

CPA-treated tubulin (5  $\mu\text{M}$ ), retyrosinated with either L-tyrosine (solid curve) or 3-formyltyrosine (dot-dash curve) and treated with coumarin hydrazine, was polymerized with paclitaxel (5  $\mu\text{M}$ ) in PMEG buffer at 37°C.



**FIGURE 6. Hydrazone formation occurs in live cells and is accompanied by an increase in probe fluorescence**

PC3 cells were grown to 50% confluence in Ham's F-10 medium. The cells were incubated for 24 h with fresh medium supplemented with 50  $\mu$ M L-tyrosine (panels A and B) or 50  $\mu$ M 3-formyltyrosine (panels C and D). After washing the cells with fresh medium, medium containing 20  $\mu$ M CH was added and the cells were observed under laser scanning confocal microscope. Panels A and C: Fluorescence images using violet laser diode excitation (405 nm) and emission bandpass of 420–480 nm. Panels B and D: Differential interference contrast images.

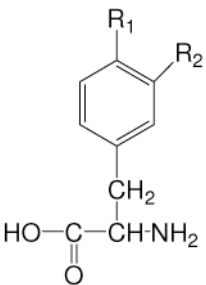


**FIGURE 7.  $\alpha$ -Tubulin is the only fluorescently-labeled protein observed in cells incubated with 3-formyltyrosine followed by coumarin hydrazine treatment**

CHO cells were incubated for 24 h in Ham's F-10 medium containing 50  $\mu$ M L-tyrosine or 50  $\mu$ M 3-formyltyrosine. The cells were washed and then treated with CH (100  $\mu$ M) for 2 h. Cells were harvested, lysed and the lysate was separated using SDS-PAGE. Panel A: Coomassie stain. Panel B: Western blot of lysate using a polyclonal antibody to  $\alpha$ -tubulin. Panel C: A gel of the lysate visualized under long wavelength UV light.

**Table 1**

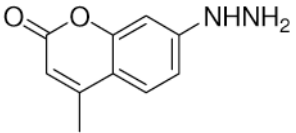
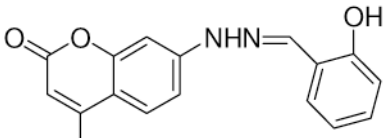
Inhibition of tubulin tyrosine ligase by tyrosine derivatives

L-Amino Acid	Structure	K <sub>i</sub> , mM	
Tyrosine	R <sub>1</sub> = OH, R <sub>2</sub> = H	0.025	
3-Nitrotyrosine	R <sub>1</sub> = OH, R <sub>2</sub> = NO <sub>2</sub>	0.4	
3-Aminotyrosine	R <sub>1</sub> = OH, R <sub>2</sub> = NH <sub>2</sub>	0.2	
3-Azidotyrosine	R <sub>1</sub> = OH, R <sub>2</sub> = N <sub>3</sub>	0.2	
3-Formyltyrosine	R <sub>1</sub> = OH, R <sub>2</sub> = CHO	0.3	
4-Aminophenylalanine	R <sub>1</sub> = NH <sub>2</sub> , R <sub>2</sub> = H	>4	



**Table 2**

Absorption and emission maxima and quantum yield of coumarin hydrazine and its salicylaldehyde hydrazone in methanol at 25 °C

fluorophore	Abs max, nm	Em max, nm	$\phi$
	360	432	0.046
	380	459	0.131