A photoaffinity derivative of colchicine: 6′-(4′-azido-2′-nitrophenylamino)hexanoyldeacetylcolchicine

PHOTOLABELING AND LOCATION OF THE COLCHICINE-BINDING SITE ON THE α-SUBUNIT OF TUBULIN*

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A photoaffinity analog of colchicine, 6′-(4′-azido-2′-nitrophenylamino)hexanoyldeacetylcolchicine, was synthesized by reacting deacetylcolchicine or [3H]deacetylcolchicine with N-succinimidyl-6′-(4′-azido-2′-nitrophenylamino)hexanoate. Homogeneity of the photoaffinity analog was established by thin-layer chromatography and high-pressure liquid chromatography. The structure of the photoaffinity analog was determined by 1H and 13C NMR, infrared and ultraviolet-visible spectroscopies, and elemental analysis. Binding of 6′-(4′-azido-2′-nitrophenylamino)hexanoyldeacetylcolchicine to bovine renal tubulin was measured by competition with [3H]colchicine. The value of the apparent Kᵢ for the photoaffinity analog was 0.28 μM in the concentration range of 0.8–1.2 μM of the analog. A value of 0.50 μM for the apparent Kᵢ was measured by the direct binding of the tritiated photoaffinity analog to tubulin. The analog is slightly more potent an inhibitor of microtubule formation than colchicine. The photoaffinity analog reacted with renal tubulin upon irradiation with a mercury lamp equipped with a 420-nm cutoff filter. Spectral and radiochemical analyses of the tubulin after photolysis and dialysis have demonstrated a stoichiometric incorporation of the photoaffinity analog in the α-subunit of tubulin. Covalent labeling of tubulin with the photoaffinity analog decreases the extent of [3H]colchicine binding by more than 90%.

Essential to understanding the reaction of colchicine with tubulin is knowledge of the properties and location of the colchicine-binding site on tubulin. Such knowledge should also facilitate elucidation of interactions among colchicine and other ligands which bind to tubulin, the role of colchicine in disassembly of microtubules, and isolation and identification of a possible endogenous ligand. Photoaffinity labeling has been used extensively in the selective labeling of receptor sites in a large variety of biological systems, especially with aryl azides as the photolabile moiety (1). Photogenerated reagents are potentially highly reactive and can be activated in situ under mild conditions. These are advantageous properties for labeling binding sites located in hydrophobic regions of proteins (2).

In this report we describe the synthesis, chemical characterization, and binding properties of 6′-(4′-azido-2′-nitrophenylamino)hexanoyldeacetylcolchicine (ANPAH-CLC), a new photoaffinity derivative of colchicine. We report also the photolabeling and subunit localization of the colchicine-binding site of renal tubulin with this analog.

Previous studies indicate that the interaction between colchicine and tubulin is primarily nonionic, noncovalent, and occurs in a hydrophobic domain of tubulin (3). Two different approaches have been taken to specifically locate the colchicine-binding site on tubulin with reactive analogs of colchicine. Bryan and co-workers synthesized two photoaffinity analogs of colchicine, but photolabeling of tubulin was unsuccessful with both analogs (4, 5). Chlorocyanoeethyl colchicine and diazomalonol colchicine were photolyzed in the presence of brain tubulin, but neither α- nor β-tubulin were labeled. Rather, a protein with a molecular weight of 16,500 was labeled, and the relationship, if any, of this protein to tubulin was unknown. Schmitt and Atlas (6) used bromocolchicine, a compound with nonspecific alkylating reactivity, to label tubulin. They concluded the colchicine-binding site was on the α-subunit, although both α- and β-subunits were labeled. Ludueña has reviewed the problems associated with such general alkylating compounds (7).

The ANPAH-CLC was prepared by reacting deacetylcolchicine (DAC) with a nitro-substituted aryl azide, N-succinimidyl-6′-(4′-azido-2′-nitrophenylamino)hexanoate (SANPAH). The structure of the derivative was established by UV-VIS, 1H and 13C NMR, and IR spectroscopic techniques. Binding experiments with renal tubulin indicated that the derivative strongly interacts with tubulin, thereby enhancing the potential for photolabeling the colchicine-binding site. Furthermore, studies on the inhibition of microtubule formation by ANPAH-CLC have shown that the analog is...

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slightly more potent an inhibitor of the tubulin polymerization than colchicine. Photolysis experiments indicate that the colchicine-binding site is located on the α-subunit of tubulin. The stoichiometry of the binding is 1:1 when the molar ratio of ANPAH-CLC to tubulin is 2:1 or less. Covalent incorporation of ANPAH-CLC into tubulin blocks the binding of [H]colchicine. Preliminary accounts of part of this work have been reported (8, 9).

EXPERIMENTAL PROCEDURES

RESULTS

Synthesis of 6-(4'-Azido-2'-nitrophenylamino)hexanoylcolchicine—The photoaffinity derivative of CLC was synthesized by acylation of the amino group of DAC with SANPAH. DAC (0.3 mol) was reacted with SANPAH (0.3 mol) in 20 ml of aceton under N₂ as shown in Equation 1. The solution was stirred for 48 h at room temperature. The reaction was monitored by TLC and the reaction was allowed to proceed until DAC was undetectable. Solvent was evaporated, and the resultant solid was dissolved in a minimum volume of methylene chloride. The mixture was purified by preparative TLC on Whatman PLK5F linear K silica gel plates (20 × 20 cm; 1000 µ) in benzene:methanol (3:1). The product was isolated by elution with methanol and recrystallized from methylene chloride-diethyl ether. The yield of ANPAH-CLC from DAC and SANPAH was 71%.

Characterization of ANPAH-CLC—ANPAH-CLC was homogenous based on TLC on silica gel in benzene:methanol (3:1) (values of RF: ANPAH-CLC, 0.5; DAC, 0.33; SANPAH, 0.95) and in methanol (values of RF: ANPAH-CLC, 0.68; DAC, 0.40; SANPAH, 0.70). ANPAH-CLC exhibited a single peak when subjected to reversed-phase HPLC. A contamination of 0.5% (w/w) of DAC or SANPAH in the purified ANPAH-CLC preparation would have been detectable by HPLC (Fig. 1A). The materials which eluted adjacent to DAC in the HPLC (Fig. 1A) were contaminants in SANPAH. ANPAH-CLC exhibited absorption maxima at 458, 350, and 250 nm with extinction coefficients of 4294, 15,150, and 33,531 M⁻¹ cm⁻¹, respectively, in 100% ethanol (Fig. 2). Absorption maxima occurred at 478, 356, and 251 nm with extinction coefficients of 7018, 15,773, and 26,633 M⁻¹ cm⁻¹, respectively, in 25 mM sodium phosphate, 1 mM MgS0₄, 0.1 mM EGTA, pH 7.2 (data not shown). Reaction of SANPAH with DAC did not change the absorption attributable to the aryl azido moiety nor was the tropolone moiety of colchicine altered (Fig. 3). Values of the absorption peaks measured by IR and the resonance frequencies measured by ¹H and ¹³C NMR confirm the structure of ANPAH-CLC as shown in Equation 1. Elemental analysis:

Calculated: C 60.85, 5.58 N 13.30
Found: C 60.03, 5.80 N 12.80

Melting point, 95-99 °C.

Synthesis of [H]ANPAH-CLC—[H]DAC (5 ml, 2 mCi/ml, 1:1 ethanol:water) was evaporated to dryness under a stream of nitrogen. A 500-µl solution of 7.61 mg (70 µmol) of SANPAH and 5 mg (13 µmol) of unlabeled DAC in aceton was added to the [H]DAC in a vial equipped with a Teflon-faced septa. To this solution 250 µl of 25 mM phosphate buffer, pH 7.5, was added. The reaction was monitored by TLC on silica (benzene:methanol, 3:1), and the TLCs were scraped and counted for radioactivity. After 4 h the DAC had been consumed and no further changes in the TLCs were observed. The reaction solution was evaporated to dryness, redissolved in HPLC-grade methanol, and partially purified by thick layer chromatography on a Whatman PLK5F linear K silica gel plate. After elution with HPLC grade methanol, the [H]ANPAH-CLC was purified to >99% by preparative HPLC on a Beckman C₁₈ reverse-phase column at a flow rate of 5.0 ml/min and a pressure of 1.70 kps.i, with a mobile phase of methanol:H₂O, 9:1. The material eluting at 20.75 ml was collected from eight injections, combined, evaporated to dryness, and reinjected on the preparative HPLC column to check purity (Fig. 1B). The [H]ANPAH-CLC was dissolved in 4.5 ml of ethanol to give a working stock solution of 6.37 × 10⁻³ M. The [H]ANPAH-CLC had a specific activity of 3.78 Ci/µmol.

Binding of ANPAH-CLC to Tubulin—ANPAH-CLC behaved as an apparent competitive inhibitor of the binding of [H]colchicine based on double-reciprocal graphical analysis of the data (Fig. 4A). However, a secondary plot of the apparent Kᵣ for colchicine versus the concentration of ANPAH-CLC was nonlinear (Fig. 4B). Secondary plots of the reciprocal of the mass of colchicine bound versus ANPAH-CLC concentration (modified Dixon plot) (24) and the slope of the primary double-reciprocal plot versus ANPAH-CLC concentration were also curved upward (data not shown). The value of the apparent Kᵣ for colchicine was 0.42 ± 0.04 µM (mean ± S.D., n = 4). The value of the apparent Kᵣ for ANPAH-CLC was 0.28 ± 0.03 µM (mean ± S.D., n = 8) in the concentration range of 0.8-1.2 µM ANPAH-CLC. [H] ANPAH-CLC bound to renal tubulin with the value of the apparent Kᵣ equal to 0.50 ± 0.04 µM (mean ± S.D., n = 4), as shown in Fig. 5.

Inhibition of Microtubule Formation—Polymerization of renal tubulin at 37 °C was initiated by addition of dimethyl sulfoxide. Microtubule formation, measured by the absorbance change at 350 nm with time, was followed in the absence and presence of various concentrations of colchicine or ANPAH-CLC. Fig. 6 shows typical data corrected for the background absorbances of Me₂SO and colchicine or ANPAH-CLC. (The absorbances of Me₂SO, CLC, and ANPAH-CLC do not change significantly upon binding (data not shown).) A 3 µM concentration of ANPAH-CLC reduced microtubule formation to a greater extent than a corresponding 5 µM...
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FIG. 4. A, binding of [3H]colchicine to renal tubulin in the absence and presence of 6-(4'-azido-2'-nitrophenylamino)hexanoyldeacetylcolchicine. Renal tubulin, 0.1 mg/ml, was incubated for 3 h at 37 °C with 0.16-3.82 pM [3H]colchicine in the absence (●) and presence of 0.4 µM (○-○), 0.8 µM (▲-▲), and 1.2 µM (△-△) ANPAH-CLC. B, values of the apparent Kd for colchicine as a function of the concentration of 6-(4'-azido-2'-nitrophenylamino)hexanoyldeacetylcolchicine. Values of the apparent Kd were calculated from slopes of primary plots of binding data (e.g. A) and are expressed as the mean ± S.D., n = 4 experiments. In the four experiments, the largest concentration of ANPAH-CLC ranged from 1.07-1.20 µM with a mean value of 1.14 µM.

The observed inhibition of tubulin polymerization by 10 µM ANPAH-CLC and 30 µM colchicine polymerization was inhibited almost completely, but other aggregation and/or precipitation of the tubulin occurred. This was similar to the aggregation effects of vinblastine that occurred at concentrations as low as 1.5 µM (data not shown). The aggregation effect was observed in the 3 µM ANPAH-CLC concentration as evidenced by the absorbance increase occurring during the latter portion of the time profile.

The observed inhibition of tubulin polymerization by 10 µM colchicine was approximately 50% of the uninhibited tubulin polymerization under the experimental conditions described in "Experimental Procedures." At concentrations over 10 times the Kd complete inhibition of polymerization would be expected providing adequate time was allowed for either colchicine or the analog to bind to tubulin. For comparison purposes between colchicine and analog the experimental protocol chosen had the required GTP present before either addition of Me2SO to a final volume of 10% in the presence or absence of various concentrations of CLC or ANPAH-CLC. Polymerization was measured by the change in absorption at 350 nm as a function of time and corrected for the absorbance increase at 350 nm due to addition of Me2SO and inhibitor. ●, control; ○-○, 5 µM CLC; ▲-▲, 3 µM ANPAH-CLC; △-△, 10 µM CLC.
tubulin affects the extent of microtubule formation. Similar results are obtained with ANPAH-CLC or other colchicine analogs (data not shown).

**Photolabeling of Tubulin with ANPAH-CLC: Spectral Analysis and Controls**—Renal tubulin and ANPAH-CLC, at a molar ratio of 1:2, were incubated together, and the UV-VIS spectrum of the solution was measured before and after irradiation. Absorbance at 475 nm decreased after irradiation due to photoreactivity of the aryl azido moiety while the absorbance at 352 nm due to the tropolone moiety was unchanged (Fig. 8). The stoichiometry of photolabeling of tubulin with ANPAH-CLC was calculated from the UV-VIS spectrum of dialyzed photolabeled tubulin (Fig. 9A), and 0.9 mol of ANPAH-CLC was incorporated per mol of tubulin. This calculation requires the assumption that the molar extinction coefficient for the tropolone moiety of ANPAH-CLC is unchanged upon incorporation into tubulin. No absorption peak at 350 nm was detected for tubulin irradiated in the presence of colchicine instead of ANPAH-CLC (Fig. 9B). The results shown in Figs. 8 and 9 demonstrate that colchicine itself is insufficient to photolabel tubulin under these photolysis conditions and that the photoreactivity of ANPAH-CLC is due to the aryl azido moiety. Photolysis of N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate in the presence of tubulin also did not show any incorporation as measured by UV-VIS absorption spectra (data not shown). Thus, both the colchicine and aryl azido moieties are required for photolabeling of tubulin.

An additional control to show that colchicine does not photolabel tubulin by itself was performed by irradiating [3H]CLC in the presence of tubulin at molar ratios of 1:1 and 2:1, respectively, with the low pressure Hg lamp in the absence of the 420-nm cutoff filter. The Hg lamp had a strong emission band at 350 nm. At the initial molar ratios of either 1:1 and 2:1 ([3H]CLC:tubulin) only 2-3% of the radiolabeled colchicine is incorporated into the tubulin. Borisy et al. (25) had previously shown that colchicine bound to tubulin, when irradiated with long wavelength UV radiation, is converted to lumicolchicine which rapidly dissociates from the tubulin, allowing the tubulin to polymerize. Our results are in agreement with these observations.

Irradiation of a 2:1 molar ratio ([3H]CLC:tubulin) with a high intensity multiple UV lamp system for 1 h showed substantial spectral changes in the colchicine; however, only 9% of the [3H]CLC was incorporated into the tubulin after this extremely vigorous treatment. Clearly, colchicine by itself under any photolysis conditions was incapable of photolabeling tubulin in a specific manner when compared to ANPAH-CLC.

**Photolabeling of Tubulin with [3H]ANPAH-CLC: Radioisotope Analysis**—Renal tubulin and [3H]ANPAH-CLC, at several different molar ratios up to 1:2, respectively, were irradiated together and subsequently dialyzed as described under "Experimental Procedures." The stoichiometry of incorporation of [3H]ANPAH-CLC into tubulin was calculated from the radioactivity and tubulin mass in the dialyzed photolabeled tubulin. The results are shown in Fig. 10. About 1 mol of ANPAH-CLC was covalently incorporated per mol of tubulin at initial molar ratios of ANPAH-CLC to tubulin up to 2:1. The mol incorporation of [3H]ANPAH-CLC into tubulin...
was 68 ± 4% (x ± S.E., n = 12) of the initial moles of the photofluorophotophore analog. Unincorporated [3H]ANPAH-CLC was photodegraded and removed by the dialysis treatment.

Colchicine Binding to Photolabeled Tubulin—Binding experiments with photolabeled tubulin provided further evidence for the specificity of ANPAH-CLC for the colchicine-binding site. Tubulin was photolabeled with ANPAH-CLC at a molar ratio of 1:2 and dialyzed. Control tubulin preparations were obtained by dialysis treatment alone and by photolysis without ANPAH-CLC followed by dialysis. The capacity of each dialyzed tubulin preparation to bind [3H]colchicine was measured as a function of the tubulin mass. The results are shown in Fig. 11. Tubulin that was photolabeled with ANPAH-CLC had only 8% of the colchicine-binding capacity of the control tubulin preparation. In addition, irradiation of tubulin alone did not significantly affect its colchicine-binding capacity.

Due to the lack of reversibility of the covalent photoincorporation of ANPAH-CLC it was impossible to remove the label and test the tubulin for general protein destruction or denaturation. Present evidence supports the thesis that the protein was not denatured or generally destroyed after photolysis; however, a conformational change in protein structure upon the photoincorporation of ANPAH-CLC could cause the observed reduction of colchicine binding rather than a specific blockage of the colchicine site. The Kᵢ of 0.28 μM for ANPAH-CLC competition of colchicine binding argues against this possibility. The experimental data suggest but do not prove that general protein destruction had not occurred.

Identification of the Photolabeled Subunit—To determine whether photosactivated ANPAH-CLC was covalently incorporated into the α- or β-subunit of renal tubulin, the photolabeled tubulin was subjected to gel electrophoresis. Initially the electrophoretic patterns of control and photolabeled tubulin samples were compared. Tubulin was photolabeled with ANPAH-CLC at a molar ratio of 1:2, dialyzed, dissociated in sodium dodecyl sulfate, and subjected to electrophoresis on a 10% acrylamide, 0.8% methylene bisacrylamide slab gel containing 0.1% sodium dodecyl sulfate. Some tubulin samples were reduced and carboxymethylated prior to electrophoresis to maximize separation of the α and β bands. Control tubulin was prepared and analyzed in the same manner except it was not irradiated. Photolabeled and control tubulin samples exhibited the same relative mobilities for the α- and β-subunits and were indistinguishable when subjected to electrophoresis together (data not shown). Thus, photolabeling of tubulin did not alter the mobilities of the α- or β-subunit. Little et al. (26) demonstrated that the α-subunit had a smaller electrophoretic mobility than the β-subunit for renal tubulin.

Tubulin was photolabeled with [3H]ANPAH-CLC at a molar ratio of 1:2, prepared for electrophoresis as described under “Experimental Procedures,” and subjected to gel electrophoresis on a 10% acrylamide slab gel containing 0.1% sodium dodecyl sulfate. The gel was stained with Coomassie Blue, destained, and then subjected to fluorography. The electrophoretic patterns of photolabeled tubulin as detected fluorographically and by protein staining are shown in Fig. 12. The [3H]ANPAH-CLC was covalently incorporated into the α-subunit of tubulin. There was no significant incorporation into the β-subunit even at the largest mass of photolabeled tubulin examined electrophoretically. This specificity of subunit photo labeling was more quantitatively measured by analysis of [3H]ANPAH-CLC-labeled tubulin after electrophoresis on hybrid gels composed of 12.5% acrylamide and 1% ME agarose. Tubulin, photolabeled with [3H]ANPAH-CLC at a molar ratio of 1:1, was subjected to electrophoresis on cylindrical hybrid gels containing 0.1% sodium dodecyl sulfate. The gels were stained with Coomassie Blue and densitometrically scanned at 610 nm (Fig. 13A). A corresponding unstained gel was sliced into 2-mm segments, the segments were melted in H₂O, and counted for tritium. The results in Fig. 13B show that more than 97% of the radioactivity was present in the α-subunit. Incorporation of [3H]ANPAH-CLC into the α-subunit of the tubulin was blocked by preincubation of tubulin with colchicine prior to photolysis. Fig. 14 shows that
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The colchicine-binding site on the α-subunit of bovine renal tubulin is based on analysis of data obtained by gel electrophoresis of [3H]ANPAH-CLC-photolabeled tubulin. This is the first time that the colchicine-binding site of tubulin has been labeled with a specific in situ-activated colchicine derivative. Our results are in agreement with previous observations on mouse brain tubulin using bromocolchicine (6) and porcine brain tubulin using limited proteolysis with [3H]colchicine binding (29) that implicate the α-subunit as the colchicine-binding site. In contrast, Ludueña and Roach found that colchicine and podophyllotoxin blocked the intrachain cross-linking of two sulfhydryl groups in brain tubulin with $N,N'$-ethylene-bisiodoacetamide (30). These sulfhydryl groups correspond to two cysteines present in the β-subunit. They interpreted these results to mean that the colchicine-binding site may be on the β-subunit (31).

Deacetylcolchicine was prepared as the initial reactant because it contains a reactive amino group as well as intact structures of rings A and C of the colchicine molecule (Equation 1). The benzenoid ring with at least one methoxy group (ring A) and the troponoid ring with specific positions for the methoxy and carbonyl moieties (ring C) result in the tight binding of CLC to tubulin (32–35). The amino-substituted moiety on ring B and even ring B itself are not considered essential (33). The nucelophilic amino group in DAC readily reacts with acids, esters, isothiocyanates, and sulfonyl chlorides for preparation of photoaffinity, spin-labeled, fluorescent, and heme-peptide derivatives of colchicine (8, 36–39).

ANPAH-CLC was synthesized as the photoactive analog because nucleophilic displacement on the $N$-succinimidyl moiety of $N$-succinimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate by the amino group of DAC yields an amide linkage that very closely resembles the structure of CLC. In addition, the highly reactive nitrene, photochemically generated from the azido group, covalently bonds by insertion into carbon-hydrogen bonds (1, 2). Thus, the reactive group is not generated until the analog binds, and the reactive group is not limited to a particular amino acid residue for covalent incorporation. The presence of the nitro group meta to the azido group shifts the wavelength maximum for photolysis to above 400 nm (1, 2, 40) and, thereby, minimizes the possibility of photochemical rearrangements of the troponoid ring of CLC.

Retention of the absorption maximum at 350 nm in ANPAH-CLC after irradiation provides a means to measure photoaffinity labeling of tubulin plus indicating that the troponoid ring is not rearranged. The data presented here support the rationale for the synthesis and application of this photoaffinity derivative of colchicine.

The free energies of binding of CLC and ANPAH-CLC to renal tubulin are about the same based on the similarity of...
values of the apparent $K_d$ for CLC and ANPAH-CLC. The similarity of values for $K_d$ is probably a reflection of the similarity of structures. The lack of linearity of secondary plots of the binding data indicates that ANPAH-CLC does not react with tubulin in a simple bimolecular reaction. Several investigators (27, 28, 41) have proposed that the binding of colchicine induces a conformational change in tubulin and that colchicine itself does not interact with tubulin in a simple bimolecular reaction. Nonlinearity of secondary plots of the binding data are a general phenomenon for CLC and colchicine analogs when a sufficiently large concentration range is examined (8, 9, 37, 38).

The inhibition of microtubule formation exhibited by the ANPAH-CLC demonstrates its ability to behave in a manner identical to colchicine. It is a slightly more potent inhibitor of microtubule formation than colchicine and at higher concentrations causes aggregation or precipitation that is analogous to the effects caused by vinblastine (42).

Knowledge of the subunit localization of the colchicine binding site on tubulin may be applicable in characterization of mutant forms of tubulin and elucidation of mechanisms of microtubule formation than colchicine and at higher concentrations causes aggregation or precipitation that is analogous to the effects caused by vinblastine (42).

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EXPERIMENTAL PROCEDURES

Materials

[4-3H]colchicine (specific activity, 7.0-6.1 Ci/mmol) was purchased from New England Nuclear and unlabeled colchicine (CLC) from Aldrich Chemical Company. The purity of [4-3H]colchicine was at least 98% as determined by TLC on silica gel G (EM Laboratories, Inc.). in chloroform:toluene:ethanol (1:4:2). TLC. CLC was purified by column chromatography on silica (Merck Kieselgel 60, 0.040-0.063 mm) followed by recrystallization from ethyl acetate-water. (benzenemethanol) (1:1) by TLC. Purified [4-3H]colchicine was checked by TLC of the solvent system previously described (12). The sample was stored in a desiccator at -20°C.

Spectral characterization of the DAC by HPLC analysis was accomplished in large scale. (by HPLC analysis) was accomplished by employing the technique of short column chromatography (13) which allows preparative separation of mixtures with a resolution equivalent to that provided by analytical TLC. Silica gel G (EM Laboratories, Inc.) was used on a Thomas Hoover Capillary melting point apparatus and was equilibrated at 37°C. The separation and purification of DAC from IDAC was accomplished in large scale by employing the technique of short column chromatography which allows preparative separation of mixtures with a resolution equivalent to that provided by analytical TLC. Silica gel G (EM Laboratories, Inc.) was used on a Thomas Hoover Capillary melting point apparatus and was equilibrated at 37°C. The separation and purification of DAC from IDAC was accomplished in large scale by employing the technique of short column chromatography which allows preparative separation of mixtures with a resolution equivalent to that provided by analytical TLC. Silica gel G (EM Laboratories, Inc.) was used on a Thomas Hoover Capillary melting point apparatus and was equilibrated at 37°C.

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Chemical characteristics

Melting points were obtained on a Thermo Haake DSC/1 differential scanning calorimeter apparatus and are uncorrected. 1H- and 13C-nuclear magnetic resonance spectra were recorded on a Bruker WP 400 spectrometer and 1H- and 13C-nuclear magnetic resonance spectra were recorded on a Bruker WM 400 spectrometer. Spectrophotometric data were recorded on a Hitachi 320 spectrophotometer. Elemental analyses were performed by Microtech Laboratories, Inc., Skokie, Illinois.
Photolabeling of Tubulin

Preparation of Tubulin

Tubulin was purified from bovine renal medulla by four cycles of 10 mM sodium phosphate, 1 mM MgCl₂, and 0.1 mM EGTA, pH 7.2, for three hours at 37°C in a stirred water bath. The assay volume was 200 μL, and the concentration of 1 mM NaCl was 0.1 μM with a specific activity of 0.25 μM. Site-directed spin-labeled (DLS) tubulin was prepared by photolysis at 37°C for 30 min. The assay was divided into two parts: one with tubulin and the other with sodium dodecyl sulfate and 15 mM sodium phosphate, pH 7.2, before the assay was started. All samples were analyzed by liquid scintillation counting of [3H]tubulin in a Beckman LS-1000 spectrophotometer.Activity of [3H]tubulin was measured by the decrease in 3H signal, which was recorded on a Beckman LS-1000 spectrophotometer.

Binding of the Photoaffinity Analog to Tubulin

Binding of the photoaffinity analog to tubulin was evaluated indirectly by competition with unlabeled colchicine. [3H]tubulin was incubated with tubulin in 0.1 M sodium phosphate, 0.1 mM EDTA, pH 6.8, for 300 μL in a water bath. The assay volume was 200 μL, and the concentration of 1 mM NaCl was 0.1 μM with a specific activity of 0.25 μM. Site-directed spin-labeled (DLS) tubulin was prepared by photolysis at 37°C for 30 min. The assay was divided into two parts: one with tubulin and the other with sodium dodecyl sulfate and 15 mM sodium phosphate, pH 7.2, before the assay was started. All samples were analyzed by liquid scintillation counting of [3H]tubulin in a Beckman LS-1000 spectrophotometer.