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SYNTHESIS AND EVALUATION OF 2-DIAZO-3,3,3-TRIFLUOROPROPANOYL DERIVATIVES OF COLCHICINE AND PODOPHYLLOTOXIN AS PHOTOAFFINITY LABELS: REACTIVITY, PHOTOCHEMISTRY, AND TUBULIN BINDING

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Abstract—Derivatives of the tubulin polymerization inhibitors colchicine and podophyllotoxin bearing the photoreactive 2-diazo-3,3,3-trifluoropropanoyl (DTFP) group were synthesized for evaluation as potential photoaffinity labels of the tubulin binding site. All labels were assayed for their ability to inhibit tubulin polymerization, and *N*-DTFP-deacetylthiocolchicine was shown to competitively inhibit tubulin-colchicine binding with a K_i of 4–5 μM. The tubulin off-rate of this analog was similar to that of podophyllotoxin, rather than to the relatively irreversibly bound colchicine. Photochemical solvent insertion reactions of the labels were investigated. Radioactive samples of the two most active labels were prepared and used in initial protein-labeling experiments, during which the fractional occupancy of tubulin and extent of covalent incorporation were determined. A rearrangement of DTFP amides was encountered which is relevant to the utility of this moiety for use in synthesis of photoaffinity labels.

INTRODUCTION

Tubulin assembly inhibitors play an important role in the study of tubulin's assembly mechanism, and of microtubule function in cell physiology (Dustin, 1984; DeBrabander and DeMey, 1985). Several approaches have been used in attempts to localize the binding site of colchicine, the most extensively studied assembly inhibitor. These include chemical modification studies (Maccioni and Seeds, 1982; Roach and Luduena; 1984; Little and Luduena, 1985), and limited proteolysis (Serrano *et al.*, 1984; Mandelkow *et al.*, 1985; Kirchner and Mandelkow, 1985). Photoaffinity methods have been pursued by Barnes and coworkers. These workers used an azidonitrophenyl group attached to deacetylcolchicine by a 6-aminohexanoyl linker arm (Williams *et al.*, 1985) and an azidonitrophenyl group attached directly to the colchicine nitrogen (Floyd *et al.*, 1989). Both α and β subunits are labeled, with the distribution depending upon the label used and the conditions of the experiment. These results have been interpreted in terms of two colchicine binding sites, with the high affinity site associated with the α subunit.

The 2-diazo-3,3,3-trifluoropropanoyl (henceforth DTFP)† group was introduced by Chowdhry *et al.* (1976) as a potential photoaffinity label and has subsequently been utilized by other investigators (Gupta *et al.*, 1979a,b; Pascual *et al.*, 1982; Schjoldager, 1988). Because it has been shown that modification of colchicine with groups closely resembling DTFP is consistent with retention of assembly inhibitory activity (Capraro and Brossi, 1984), we have synthesized DTFP derivatives of colchicine and evaluated their potential as photoaffinity labels for the colchicine binding site on tubulin. A colchicine derivative incorporating two DTFP groups was also prepared. Since it has been suggested that colchicine may bind in a cleft between the α and β subunits (Little and Luduena, 1985; Lin *et al.*, 1989), a bifunctional label could conceivably crosslink the tubulin subunits. A photoaffinity derivative of podophyllotoxin, a potent assembly inhibitor whose binding site overlaps that of colchicine (Cortese *et al.*, 1977; Loike *et al.*, 1978), was also synthesized and evaluated.

The polymerization inhibition activity of each photoaffinity label was assayed as an initial screen for binding to the colchicine-binding site. One of the analogs was shown to competitively inhibit colchicine binding, and its tubulin binding kinetics were examined. The chemical and photochemical reactivity of the analogs were tested to formulate appropriate conditions for tubulin labeling. The extent of

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†*Abbreviations*: CIMS, chemical ionization mass spectrometry; CPM, counts per minute; DTFP, 2-diazo-3,3,3-trifluoropropanoyl; DTFP-Cl, 2-diazo-3,3,3-trifluoropropanoyl chloride; EIMS, electron impact mass spectrometry; FABMS, fast atom bombardment mass spectrometry; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance; PMEG buffer, 100 m*M* PIPES, 1 m*M* $MgSO_4$, 2 m*M* EGTA, and 0.1 m*M* GTP, pH 6.9; PMR, proton magnetic resonance and TLC, thin layer chromatography.

covalent protein labeling by the two most active analogs was determined.

MATERIALS AND METHODS

General. Synthetic intermediates and photoaffinity labels were purified by flash column chromatography using EM Reagents kieselgel 60 (230–340 mesh) silica gel as described by Still *et al.* (1978). Purity was determined by TLC carried out with EM Reagents pre-cut TLC plates, kieselgel 60 F-254. PMEG buffer was a solution of 0.1 *M* PIPES, 1 m*M* $MgSO_4$, 2 m*M* EGTA, and 0.1 m*M* GTP in distilled water, adjusted to pH 6.9 with sodium hydroxide. All buffers were filtered through Millipore filter HAWP 04700, 0.45 μm. PMEG components, 2-mercaptoethanol, iodoacetic acid, trypsin, and dithiothreitol were purchased from Sigma Chemical Company, (St. Louis, MO), and ^3H_2O was obtained from the New England Nuclear Co. (Boston, MA), Sephadex G-50 (fine) was purchased from the Pharmacia Co. (Piscataway, NJ), and unless otherwise noted was equilibrated with PMEG buffer prior to use. Dialysis was with Spectrapor dialysis membrane tubing of 12000–14000 molecular weight cutoff. Scintillation counting of aqueous solutions was performed in 10 mL of Amersham ACS scintillation cocktail.

The following compounds were prepared using known methods, modified as described in the doctoral dissertation of Hahn (1987): 2-diazo-3,3,3-trifluoropropanoyl chloride (DTFP-Cl) (Gilman and Jones, 1943; Chowdhry *et al.*, 1976; Radhakrishnan *et al.*, 1981), allocolchicine (Fernholz, 1949), deacetyl-allocolchicine (Deinum *et al.*, 1981), 3-demethylcolchicine (Rossner *et al.*, 1981), 2-demethylthiocolchicine (Sharma and Brossi, 1983).

Synthesis of the following compounds is described in the doctoral dissertation of Hahn (1987): 10-(2-hydroxyethyl)thiocholchicine and its thiolane derivative, 2-(2-diazo-3,3,3-trifluoropropanoyloxy)-2-demethoxythiocolchicine (label **A2**), 3-(2-diazo-3,3,3-trifluoropropanoyloxy)-3-demethoxycolchicine (label **A3**), 10-[2-(2-diazo-3,3,3-trifluoropropanoyloxy]ethyl) thiocolchicine (label **C1**), 3-(2-diazo-3,3,3-trifluoropropanoyloxy)-(*N*-2-diazo-3,3,3-trifluoropropanoyl)-3-demethoxy-*N*-deacetylthiocolchicine (label **AB-Bis**), (*N*-2-diazo-3,3,3-trifluoropropanoyl)-*N*-deacetylallocolchicine (Allo label), (*O*-2-diazo-3,3,3-trifluoropropanoyl)-podophyllotoxin (Podo label). 2-demethylthiocolchicine-4-^{3}H, 2-(2-diazo-3,3,3-trifluoropropanoyl)-2-demethoxythiocolchicine-4-^{3}H (**A2-t**).

Thiocolchicine and deacetylthiocolchicine. These compounds were produced using the procedures of Shiau *et al.* (1975), modified for use on a small reaction scale. Thiocolchicine was dissolved in a 1:1 methanol:2 *N* HCl solution and refluxed under nitrogen in the dark until TLC (8:2 ethyl acetate:ethanol) indicated the disappearance of starting material (about 40 h). The reaction mixture was evaporated to half volume, brought to pH 13 with 50% NaOH solution. The product was extracted with chloroform and purified by flash column chromatography using 8:2 ethyl acetate:ethanol as the eluting solvent.

Thiocolchifoline. The procedure for synthesis of this previously unknown compound was derived from that of Iorio *et al.* (1981) for preparation of colchifoline, using deacetylthiocolchicine as a starting material. The reaction was monitored by TLC on silica using 1:1 ethyl acetate:ethanol. IR 3200–3700, 2920, 1680, 1540, 1350, 1320, 1200, 1130, 1090, 1020, 800, 720 cm^{-1}. PMR 7.50 (1H, d, J = 7.2), 7.32 (1H, d, J = 10.4), 7.32 (1H, s), 7.11 (1H, d, J = 10.4), 6.55 (1H, s), 4.70 (1H, m, J = 6.5), 4.13 (1H, d, J = 16.6), 4.02 (1H, d, J = 16.6), 3.94 (3H, s), 3.91 (3H, s), 3.65 (3H, s), 2.65–1.95 (4H, complex overlapping multiplets), 2.44 (3H, s).

N-(2-Diazo-3,3,3-trifluoropropanoyl)cyclohexylamine. Cyclohexylamine (70 mg) was dissolved in 10 mL dry dichloromethane and cooled in an ice bath with constant stirring. DTFP-Cl (40 mg) was dissolved in 3 mL dry dichloromethane and slowly added to the cyclohexylamine solution. The resulting mixture was stirred at room temperature for 4 h, then washed with 5% HCl followed by saturated NaCl, dried over sodium sulfate and evaporated. IR 3280, 2900, 2810, 2100, 1630, 1520, 1330, 1100, 1050 cm^{-1}. UV (in 95% EtOH) 218 nm, ϵ = 9,700.

*Thiocolchicine photoaffinity label (label **B1**).* Deacetylthiocolchicine, 50 mg, was dissolved in 5 mL of dry dichloromethane. The stirred solution was cooled in an ice bath, and 1 equiv of triethylamine and 0.2 equiv of 4-(dimethylamino)pyridine were added. A solution of 2 equiv of DTFP-Cl in 5 mL of dry dichloromethane was then added slowly over 10 min. When TLC (silica, 1:1 hexane:ethyl acetate) showed the completion of the reaction, the mixture was allowed to warm to room temperature. It was washed with ice cold 10% HCl solution, concentrated Na_2CO_3 solution, and water. Drying over Na_2SO_4 and evaporation yielded 80% of **B1** contaminated with a trace of diacylated material. Pure product was obtained by flash column chromatography using 1:1 ethyl acetate:hexane as the eluting solvent. IR (in CCl_4) 3200–3400, 2920, 2110, 1610, 1670, 1530, 1560, 1330, 1350, 1140 cm^{-1}; UV (95% ethanol) 253 nm ϵ 21200, 290 nm ϵ 9740, 388 nm ϵ 14800. EIMS *m/e* 509, 481. PMR 7.29 (1H, d, J = 10.8), 7.21 (1H, s), 7.04 (1H, d, J = 10.8), 6.54 (1H, s), 6.35 (1H, broad), 4.71 (1H, m, J = 6.5 apparent), 3.94 (3H, s), 3.91 (3H, s), 3.66 (3H, s), 2.65–1.85 (4H, complex overlapping multiplets), 2.44 (3H, s).

*Thiocolchifoline photoaffinity label (label **B2**).* Thiocolchifoline, 30 mg, was dissolved in 7 mL dry dichloromethane. While the mixture was stirred and cooled with an ice bath, 1 equiv of triethylamine and 0.2 equiv of 4-(dimethylamino)pyridine were added. A solution of 2 equiv of DTFP-Cl in 5 mL of dry dichloromethane was then added slowly over 10 min. When TLC (silica, ethyl acetate) showed completion of the reaction, the product was isolated as described for **B1** (80–90% yield). IR 3200–3350, 3010, 2980, 2100, 1710, 1690, 1540, 1310, 1340, 1260, 1120, 1080, 1000 cm^{-1}. UV (95% ethanol) 238 nm ϵ 26300, 250 nm ϵ 22000, 290 nm ϵ 11500, 387 ϵ 17100. CIMS *m/e* 568, 540, 512, 384, 357. PMR 7.23 (1H, d, J = 10.5), 7.14 (1H, s), 6.98 (1H, d, J = 10.5), 6.46 (1H, s), 4.63 (1H, d, J = 16.2), 4.61 (1H, m, J = 6.5), 4.53 (1H, d, J = 16.2), 3.87 (3H, s), 3.83 (3H, s), 3.58 (3H, s), 2.52–1.72 (4H, complex overlapping multiplets), 2.36 (3H, s).

Tritium labeled deacetylthiocolchicine. Thiocolchicine (50 mg) in methanol (50 mL) was added to a glass pressure tube approx. 1.25 cm in diameter, sealed at one end. Acetyl chloride (212 μL) was carefully added to 1 mL of ^3H_2O. The water/acetyl chloride mixture (50 μL) was added to the pressure tube and the mixture was shaken to solubilize thiocolchicine. The tube was frozen in a dry ice/acetone bath. Methanol (50 μL) was added and used to flush solid from the side to the bottom of the tube. The tube was sealed and placed in a pressure bottle about one-third filled with distilled water. The pressure bottle was sealed and heated at 80°C for 46 h, after which it was cooled and opened, and the contents of the tube were transferred to a separatory funnel. The tube was washed several times with water, which was added to the separatory funnel. The reaction mixture was made basic with cold 10% NaOH and extracted exhaustively with dichloromethane. The dichloromethane was dried over Na_2SO_4, transferred to a beaker and allowed to evaporate to dryness. The crude solid was purified by flash column chromatography (silica, 8:2 ethyl acetate:ethanol). Both deacetylthiocolchicine (65–80% yield) and thiocolchicine were recovered. After purification by flash chromatography, the specific activity of both products was 7×10^{12} cpm/mol.

N-(2-Diazo-3,3,3-trifluoropropanoyl) deacetylthiocolchicine-4-^{3}H, (B1-t). This reaction was carried out using the procedure for synthesis of **B1**, but starting with tritiated deacetylthiocolchicine. Tritiated deacetylthiocolchicine (17 mg) was reacted with triethylamine (4.6 mg, 1 equiv), 4-(dimethylamino)pyridine (1.4 mg, 0.25 equiv), and freshly prepared DTFP-Cl (15.6 mg, 2 equiv). TLC (silica, 1:1 ethyl acetate:hexane) showed the reaction to be very clean. The reaction mixture was diluted with the dichloromethane, washed with ice cold 5% HCl, then with water, and dried over Na_2SO_4. All washes were backwashed with dichloromethane. The product was purified by flash column chromatography (silica, 1:1 ethyl acetate:hexane). An 82% yield (19 mg) of TLC pure **B1-t** was isolated. The specific activity was 7×10^{12} cpm/mol.

O-(2-Diazo-3,3,3-trifluoropropanoyl) thiocolchifoline-4-^{3}H, (B2-t). This compound was prepared from tritiated N-deacetyl-thiocolchicine using the synthesis described for nonradioactive **B2**.

***B1** rearrangement reaction.* Label **B1** was found to rearrange under a variety of reaction conditions. Under some of these conditions a lesser amount of a second material was also produced. TLC (silica, 8:2 ethyl acetate:ethanol and 1:1 ethyl acetate:hexane) showed both products to be much more polar than starting material and to have similar R_f values. The minor product had a slightly higher R_f than the major product. A small amount of **B1** (<5 mg) was dissolved in the following solvents and the reaction was monitored by TLC after 24 h; (1) dry dichloromethane: no reaction; (2) 25% acetonitrile in water: traces of both products: (3) 25% acetonitrile in PMEG buffer: approx. 50% conversion to lower R_f product, with very faint trace higher R_f material; (4) potassium phosphate buffer, pH 6.9: Approximately 50% conversion to equal amounts of the two products.

In a number of preparative trials, **B1** was reacted with triethylamine in dry dichloromethane. This produced the lower R_f product in nearly quantitative yield with no trace of other products. In a typical run, 22 mg **B1** was dissolved in 10 mL dichloromethane in a 25 mL flask. Triethylamine (2–3 drops) was added and the reaction was stirred for 4 h at room temperature. The reaction mixture was washed with 5% HCl and dried over Na_2SO_4. The product was purified by flash column chromatography (silica, 8:2 ethyl acetate: ethanol) to yield nearly 95% pure rearrangement product. When isopropylamine was substituted for triethylamine in this reaction the same product resulted. The product was recrystallized from methanol/water or from ethyl acetate.

Spectra of the major product: PMR (DMSO) 7.26 (1H, d, J = 10.4), 7.17 (1H, d, J = 10.4), 6.84 (1H, s), 6.44 (1H, broad, s, sharpens on increase of temperature), 4.83 (1H, d of d, J = 5.4, 11.2), 3.85 (3H, s), 3.80 (3H, s), 3.55 (3H, s), 2.7–2.84 (2H, overlapping multiplets), 2.39 (3H, s), 2.3–2.45 (1H, multiplet), 2.05–2.2 (1H, multiplet). PMR in $CDCl_3$ shows the features of the DMSO spectrum, but all peaks are broadened to differing degrees. Proton decoupled ^{13}C-NMR (DMSO) to the nearest 0.5: 181.0, 157.5, 153.5, 151.0, 150.0, 141.0, 137.0, 134.5, 129.0, 125.5, 108.0, 61 (2 peaks, close together), 56, 54.5, 34.5, 29.5, FABMS 510 (M+1). IR 3400, 2900, 1580 cm^{-1}. UV 386 ε 15200 (max.), 318 ε 4500 (min.), 290 ε 10200 (max.), 256 ε 21000 (max.).

Tubulin preparation. Tubulin was prepared essentially as described by Williams and Lee (1982), based on procedures originated by Shelanski *et al.* (1973) and Weingarten *et al.* (1975). The Williams and Lee procedure was performed with slight modifications as described in the doctoral dissertation of Bane (1983).

Tubulin concentration determination. Tubulin concentration was determined using the protein assay of Bradford (1976) as calibrated for tubulin by Bane (1983). The tubulin extinction coefficient at 250 nm was calculated as 0.69 mL mg^{-1} cm^{-1}, using the reported tubulin extinction coefficient at 278 nm (Detrich and Williams, 1978), and the observed ratio of tubulin 278 nm and 250 nm absorbance.

Polymerization inhibition assay. The assay was performed using the method of Gaskin *et al.* (1974). Solutions of inhibitor and 1 mg/mL tubulin were incubated for 30 min at 25°C prior to addition of GTP for initiation of polymerization. The final extent of polymerization, indicated by a plateau in turbidity, was recorded. All 50% inhibition values were determined by using linear least squares analysis to fit a line to all points which correspond to inhibition values other than 0 or 100%.

*Colchicine–**B1** competitive binding.* The ability of **B1** to inhibit the binding of ^{3}H–colchicine to tubulin was assessed by a modification of the filter disk assay routinely employed in investigations of the binding of colchicine analogs to the colchicine site on tubulin (Zweig and Chignell, 1973). The deviation from these procedures occurred in the separation of protein bound ligand from unbound ^{3}H–colchicine, in which 1 mL Sephadex G-50 (fine) columns were employed due to their greater ease and reproducibility. This method involves incubation of the ligand to be tested and ^{3}H–colchicine as previously described (Zweig and Chignell, 1973), then application of aliquots of the incubation mixture (up to 100 μL) to a column, centrifugation for 2 min at 900 **g** and analysis of the effluent for tubulin bound ^{3}H–colchicine by scintillation spectrometry. Control experiments determined the amount of ^{3}H–colchicine in the effluent to be negligible.

*Reversibility of **B1**–tubulin binding.* Sephadex columns were run using the centrifugation procedure of Penefsky (1979). Pharmacia G-50 fine Sephadex preequilibrated with PMEG buffer was used. Tubulin concentrations were determined using the procedure of Bradford described above. Colchicine, **B1**, podophyllotoxin, and ^{3}H–thiocolchicine were dissolved separately in 18% (vol/vol) DMSO in PMEG buffer. The concentrations of the solutions were determined by measuring their UV absorbance. Phosphocellulose purified tubulin was passed through 1 mL Sephadex columns and the concentration of tubulin in the eluent was found to be 5.3 mg/mL. The following five procedures were carried out simultaneously in duplicate: (A) to 190 μL of the tubulin solution (1 mg tubulin) were added 7.2 μL colchicine stock solution (3 equiv colchicine). The mixture was gently vortexed and incubated 20 min at room temperature. It was then passed through a Sephadex column and 32 μL ^{3}H–thiocolchicine stock (3 equiv ^{3}H–thiocolchicine) was added with gentle vortexing. After 10 min at room temperature the solution was diluted 1 in 5 with PMEG buffer, again with gentle vortexing. At varying times 200 μL aliquots were removed and passed through Sephadex. One hundred microlitres of the eluent was then counted for radioactivity; (b) the same operations were carried out with label **B1**; (c) podophyllotoxin; (d) ligand-free tubulin; and (e) 190 μL of PMEG buffer in place of tubulin. Aliquots were removed for counting at 30, 55, 105, 130, and 160 min.

Irradiation device. A 6 × 13 cm cylinder of optosil 1 quartz was filled with the solution filter, described below. A large rubber stopper with several holes was inserted in the open end of the cylinder. The rubber stopper held in place two quartz test tubes measuring 1.3 × 13 cm, or four quartz test tubes measuring 0.4 × 11 cm. The cylinder was strapped to the outside of a water cooled quartz immersion well holding a mercury arc lamp (Hanovia 679A0360). The test tubes were positioned in the rubber stoppers at a depth giving maximum irradiation of solutions in the tubes and in a position to maximize the light path through the solution filter. The lamp/cylinder array was placed inside a large tub filled with ice water. After a flow of cooling water was established in the immersion well, the reaction mixture was placed in the test tubes, the tubes were sealed with septa, and irradiation was

begun. The temperature of the filter solution during irradiation was measured periodically by inserting a thermometer through a hole in the rubber stopper. The temperature was held below 12°C throughout the run by periodic addition of ice.

Solution filter. The components of the solution filter were chosen based on information in Murov's *Handbook of Photochemistry* (1973). 2,7-Dimethyl-3,6-diazacyclohepta-2,6-diene perchlorate was synthesized using the procedure of Lloyd *et al.* (1966). An aqueous solution of 500 g/L nickel sulfate hexahydrate and 0.2 g/L 2,7-dimethyl-3,6-diazacyclohepta-2,6-diene perchlorate was prepared. It was preirradiated for 3 h and used as the solution filter.

*Irradiation of **B1** in methanol.* Label **B1** was dissolved in methanol sparged with nitrogen. This solution was placed in the irradiation device (with the solution filter) in two quartz test tubes, under nitrogen. Irradiation was begun and the reaction was monitored by TLC. The starting material was completely converted to two products with closely spaced R_fs higher than that of starting material. The products were isolated by preparative TLC (silica, 1:1 ethyl acetate:hexane). 3.3 mg of the higher R_f material and 2.2 mg of the lower R_f material were isolated. Spectral characterization showed them to be two diastereomers resulting from insertion into methanol.

Spectra of higher R_f methanol insertion diastereomer: IR 3400, 2960, 1700, 1640 cm^{-1}. EIMS *m/e* = 513, 485, 452. PMR 7.32 (1H, d, J = 10.8), 7.13 (1H, d, J = 7.6), 7.10 (1H, s), 6.55 (1H, s), 4.70 (d of t, J = 6.8, 11.1), 4.03 (1H, quartet, J = 6.8), 3.95 (3H, s), 3.92; (3H, s), 3.76 (3H, s), 3.64 (3H, s), 2.53–2.61 (1H, d of d, J = 7.2, 14.4), 2.45 (3H, s), 2.40–2.50 (1H, multiplet), 2.45 (3H, s), 2.2–2.35 (1H, multiplet), 1.83–1.93 (1H, multiplet).

Spectra of lower R_f methanol insertion diastereomer: IR 3420, 2980, 1690, 1620. EIMS *m/e*, 513, 485, 452. PMR 7.28 (1H, d, J = 11.2), 7.16 (1H, d, J = 7.2), 7.09 (1H, d, J = 11.12), 6.54 (1H, s), 4.64 (1H, d of t, J = 6.8, 11.2), 3.98 (1H, quartet, J = 7.2), 3.95 (3H, s), 3.91 (3H, s), 3.66 (3H, s), 3.64 (3H, s), 2.54–2.60 (1H, d of d, J = 6.8, 14.4), 2.43 (3H, s), 2.4–2.52 (1H, multiplet), 2.2–2.35 (1H, multiplet), 1.85–1.96 (1H, multiplet).

Irradiation of allocolchicine label. Allocolchicine label (10 mg) in 44 mL dry, distilled methanol was irradiated for 1 h at 254 nm with a mercury arc lamp. The reaction was monitored by TLC on silica using 1:1 ethyl acetate: hexane, 7:3 ethyl acetate:hexane, hexane, and 8:2 ethyl acetate:ethanol. Formation of two major products and traces of four other materials was observed. The major products were isolated using chromatography on silica. A crude separation was first effected by eluting with 1:1 ethyl acetate:hexane, followed by 9:1 ethyl acetate: ethanol. The mixtures thus isolated were separated into pure compounds by further chromatography as follows: first eluted mixture further chromatographed with 5:3 ethyl acetate:hexane, second eluted mixture with 9:1 ethyl acetate:ethanol.

Diastereomer 1: CIMS *m/e* 526, 498, 466, 344. PMR (chem shifts values separated by "/" are from protons showing doubling due to resolution of diastereomers, amide rotamers, or atropisomers) 8.00/8.06 (1H, d of d, J = 1.5, 7.6), 8.01/7.93 (1H, broad s), 7.67/7.58 (1H, d, J = 7.6), 7.06/6.70 (1H, d, J = 7.2), 6.59/6.67 (1H, s), 5.30/4.83 (1H, multiplet), 4.04/3.75 (1H, q, J = 6.5), 3.95–3.90 (3H, fine splitting), 3.95–3.90 (6H, fine splitting), 3.67/3.56 (3H, s), 3.54/3.24 (3H, s), 1.89–2.56 (4H, complex overlapping multiplets).

Diastereomer 2: CIMS *m/e* 526, 498, 466, 344. PMR (chem shifts values separated by "/" are from protons showing doubling due to resolution of diastereomers, amide rotamers, or atropisomers) 8.02/7.96 (1H, d of d, J = 7.8, 2.8), 7.98/7.92 (1H, broad, s), 7.8/7.56 (1H, d), 7.07/6.44 (1H, d, J = 7.5), 6.64/6.56 (1H, s), 5.19/4.93 (1H, t, J = 7.0), 4.06 (1H, q, 6.5), 3.90–3.95 (3 singlets, each 3H), 3.80/3.67 (3H, s), 3.27/3.54 (3H, s), 1.85–2.55 (4H, complex overlapping multiplets).

Protein irradition and dialysis. Phosphocellulose-purified tubulin was thawed and immediately passed through Sephadex columns to bring the tubulin into PMEG buffer (1–8 mg was usually used). The eluate was placed on ice, its volume was measured, and its tubulin concentration was determined by the Bradford method (see above). The desired number of label equivalents was added as rapidly as possible and the solution was vortexed briefly. The test tube containing the solution was filled with nitrogen, sealed with parafilm and incubated at 37°C for 1 h. After incubation, the tubulin-label solution was subjected to Sephadex chromatography through short columns by the centrifugation method of Penefsky (1979). As rapidly as possible, the tubulin concentration was determined and aliquots were removed for scintillation counting. The tubulin-label solution volume was measured and it was placed in the irradiation device. The tubes were filled with nitrogen and sealed. The temperature was measured during or immediately after irradiation and never exceeded 12°C. The irradiated tubulin-label solution was placed in dialysis tubing and dialyzed at 4°C against 1L of 4 mg/mL NH_4CO_3 solution. This solution was changed at least twice and dialysis after each change of solution was carried out for minimum of 10 h. After dialysis the fluid in the tubing was collected and its volume was measured. Aliquots were removed for scintillation counting.

Tubulin reduction and carboxymethylation. A procedure adapted from Perham (1978) was used. Tubulin was dissolved in fresh 4 mg/mL NH_4CO_3. A concentration as close as possible to 10 mg/mL was prepared by dialyzing the protein against aqueous NH_4CO_3, lyophilizing to dryness, and redissolving in the correct amount of buffer. Twenty microlitres of fresh 0.2 M aqueous dithiothreitol solution per mL of protein solution was added. The reaction vessel was flushed immediately with nitrogen, sealed, and incubated for 1 h or more at 30° under nitrogen. Twenty microlitres per mL fresh 0.5 *M* aqueous iodoacetic acid solution and enough solid NH_4CO_3 to bring the pH above 8 were added. The reaction vessel was immediately flushed with nitrogen and the solution was incubated in the dark for 0.5 h. One drop of β-mercaptoethanol was added to destroy unreacted iodoacetic acid and the mixture was lyophilized to dryness.

Trypsin digestion. Tubulin was dissolved to a concentration of 10–20 mg/mL in fresh 4 mg/mL NH_4CO_3 solution. Trypsin was added as a 1 mg/mL solution, at 1% of the tubulin by weight. The mixture was incubated under nitrogen at 37°C for a minimum of 3 h. Digestion was found to be most effective when the protein was first digested, then reduced and carboxymethylated, and finally digested again. This usually left very little material insoluble in aqueous NH_4CO_3.

High performance liquid chromatography conditions. The HPLC gradients used for separation of tubulin tryptic digests were as follows. Alkaline gradient: 0–40% acetonitrile in NH_4CO_3 buffer over 60 min; 40–70% acetonitrile in ammonium bicarbonate buffer over an additional 15 min; 70–0% acetonitrile in ammonium bicarbonate buffer over an additional 10 min; Acidic gradient: increasing 10–80% acetonitrile in 0.05% aqueous trifluoroacetic acid over 60 min. The NH_4CO_3 buffer was 0.05 *M* NH_4CO_3 adjusted to pH 7.3–7.4 with glacial acetic acid. All buffers were made up immediately prior to use. A 15 cm C-18 reversed phase column with a flow rate of 0.5 mL/min was used for protein loads of approx. 0.1–1 mg. A 30 cm semipreparative column at a flow rate of 1.5 mL/min was used with protein loads of approx. 1–3 mg. Absorbance was monitored at 215 nm. Aliquots were removed from each HPLC fraction and their radioactivity was determined by scintillation counting to calculate the recovery of label.

RESULTS

Synthesis and characterization of photoaffinity labels

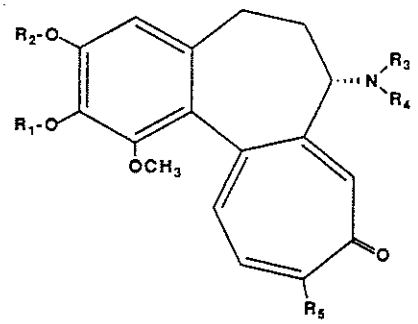
The DTFP group can be introduced *via* the reactive acylating agent 2-diazo-3,3,3-trifluoropropanoyl chloride (Chowdhry *et al.*, 1976). To accommodate this reactivity, thiocolchicine derivatives with nucleophilic sites were prepared. The thiocolchicine system was chosen because, in contrast to the 10-methoxy group in colchicine, the methylthio substituent is stable to reaction conditions which hydrolytically remove the *N*-acetyl group from colchicine (Shiau *et al.*, 1975). The known thiocolchicine derivatives *N*-deacetylthiocolchicine (Iorio *et al.*, 1981), 2-demethylthiocolchicine (Sharma and Brossi, 1983), and 3-demethylcolchicine (Rossner *et al.*, 1981) were prepared. Thiocolchifoline was prepared from *N*-deacetylthiocolchicine following a sequence analogous to that reported for colchifoline (Iorio *et al.*, 1981). A new colchicine derivative incorporating a β-mercaptoethylthio group at C-10 was prepared to provide a nucleophilic substituent on the C-ring.

All the photoaffinity analogs were characterized by NMR, IR, and mass spectrometry. In every case the spectra were consistent with those expected for the assigned structures. The purity of the samples was also demonstrated by TLC. Some of the compounds are moderately unstable to long term storage (*vide infra*) and samples used in biological assays were repurified by chromatography as necessary.

^{3}H(4)–Deacetylthiocolchicine was synthesized in 65–80% yield by heating thiocolchicine in a solution of methanol, water, tritium-enriched water, and acetyl chloride. Thiocolchicine and the **B1-t** and **B2-t** synthesized from the reaction product were obtained with specific activities of 7.2×10^{12} cpm/mol. The position of substitution was confirmed by substitution of deuterated for triated water under the conditions of the exchange reaction. Analysis of the product by proton NMR, revealed 40–50% substitution of the C-4 proton by deuterium. ^{3}H(4)–2-Demethylthiocolchicine was produced by dissolving 2-demethylthiocolchicine in acetonitrile, adding 20 equiv of separately prepared *O*-[^{3}H]trifluoroacetic acid, and heating in a sealed tube. Reaction of the product with DTFP-Cl led to the formation of **A2-t** with a specific activity of 3.2×10^{12} cpm/mol. Substitution of deuterated for tritiated water in this experiment revealed the replacement of 30% of the C-4 proton by NMR.

To demonstrate that the tritium in the labels would be stable to the aqueous solutions used in photoaffinity labelling experiments, colchicine was dissolved in 160 n*M* trifluoroacetic acid in deuterated water and monitored for 17 days. No exchange

Table 1. Structures of natural products, nucleophilic derivatives, and photoaffinity labels



	R_1	R_2	R_3	R_4	R_5
Colchicine	$-CH_3$	$-CH_3$	$-COCH_3$	$-H$	$-OCH_3$
Podophyllotoxin	$-H$	*	*	*	*
2-Demethylcolchicine	$-H$	$-CH_3$	$-COCH_3$	$-H$	$-OCH_3$
3-Demethylcolchicine	$-CH_3$	$-H$	$-COCH_3$	$-H$	$-OCH_3$
Deacetylthiocolchicine	$-CH_3$	$-CH_3$	$-H$	$-H$	$-SCH_3$
Hydroxyethylthiocol.	$-CH_3$	$-CH_3$	$-COCH_3$	$-H$	$-SCH_2CH_2OH$
Thiocolchifoline	$-CH_3$	$-CH_3$	$-COCH_2OH$	$-H$	$-SCH_3$
Allocolchicine	$-CH_3$	$-CH_3$	$-COCH_3$	$-H$	*
Label **A2**	$-$DTFP	$-CH_3$	$-COCH_3$	$-H$	$-SCH_3$
Label **A3**	$-CH_3$	$-$DTFP	$-COCH_3$	$-H$	$-SCH_3$
Label **B1**	$-CH_3$	CH_3	$-$DTFP	$-H$	$-SCH_3$
Label **B2**	CH_3	$-CH_3$	$-COCH_2O$(DTFP)	$-H$	$-SCH_3$
Label **C1**	$-CH_3$	$-CH_3$	$-COCH_3$	$-H$	SCH_2CH_2O(DTFP)
Label **AB-bis**	$-$DTFP	$-CH_3$	$-$DTFP	$-H$	$-SCH_3$
Label **B1-bis**	$-CH_3$	$-CH_3$	$-$DTFP	$-$DTFP	$-SCH_3$
Allo label	$-CH_3$	$-CH_3$	$-$DTFP	$-H$	*
Podo label	$-$DTFP	*	*	*	*

of deuterium for colchicine protons was seen under these conditions.

Tubulin binding properties

The photoaffinity analogs were evaluated for activity as tubulin assembly inhibitors using an adaptation of the turbidometric method of Gaskin *et al.* (1974). I_{50} values, which represent the concentration of ligand required to inhibit assembly to 50% of the control, are shown in Table 2. These studies revealed that **B1** and **B2** are the most active analogs and have $I_{50} < 10\ \mu M$. Labels **A2**, **A3**, and **B1-bis** have $I_{50} \approx 10\ \mu M$.

The effects of label **B1** on the binding of tritiated colchicine to tubulin were examined. The results of these experiments, shown in Fig. 1, indicate a competitive binding interaction between the two ligand (K_i = 4–5 μM), providing further evidence that **B1** does bind to the colchicine binding site on tubulin.

Assembly inhibitors of the colchicine family have been classified as "reversible" and "irreversible" on the basis of the kinetics of the tubulin binding process. Colchicine exhibits the irreversible type of behavior (Garland, 1978; Lambeir and Engelborghs, 1981). It is bound rather slowly ($k \approx 10^2$ M^{-1} s^{-1} at 37°C) but once bound the rate of dissociation is very slow. This is attributed to a mutually induced conformational change in both colchicine and the protein (Bane *et al.*, 1984). Some colchicine derivatives and podophyllotoxin exhibit reversible binding. Both the on and off rates are much faster than for colchicine, but the K_{eq} for binding is of the same magnitude (Ray *et al.*, 1981; Andreu *et al.*, 1984). For purposes of photoaffinity studies the property of being "irreversibly" bound is desirable, as it minimizes problems associated with photolysis of reversibly dissociated label molecules which may become covalently attached to protein in a non-selective manner.

To assess the reversibility of **B1** binding, the rates at which tritiated colchicine displaced bound **B1**, colchicine, and podophyllotoxin were compared. The rate of tritiated thiocolchicine binding to free tubulin was also measured as a control. Results of these experiments, presented in Fig. 2, show that the off rate of **B1** is much closer to that of the reversibly bound podophyllotoxin than to that of "irreversibly" bound colchicine. Podophyllotoxin has been shown to dissociate from tubulin with a half life of 22 min at 37°C (Cortese *et al.*, 1977), and the colchicine off rate has been measured at 5–9 × 10^{-6} s^{-1} at 37°C (Garland, 1978).

Table 2. I_{50} Values of tubulin polymerization inhibitors

Inhibitor	I_{50} (μM)
Colchicine	1.6
Podophyllotoxin	1.7
Label **A2**	9.6
Label **A3**	10.8
Label **B1**	5.5
Label **B2**	2.5
Label **C1**	85.6
Label bis-**B1**	9.0
Label **AB**-bis	26.3
Allo label	25.0
Podophyllotoxin label	5.9

The I_{50} concentration is the inhibitor concentration producing half maximal tubulin polymerization.

Photochemistry and chemical stability

The expected photolytic reactivity of the DTFP group is the generation of a carbene capable of undergoing insertion reactions (Chowdhry *et al.*, 1976). *N*-DTFP cyclohexylamine was synthesized to obtain a UV absorption spectrum of the isolated DTFP chromophore. This spectrum, shown in Fig. 3, made it clear that the major portion of the DTFP absorbance occurs between 200 and 250 nm. A potential complication arises from the strong UV absorption of the thiocolchicine C ring chromophore at 350 nm. The photochemistry of thiocolchicine has not been investigated, but colchicine's C ring undergoes a well-characterized series of intramolecular photochemical reactions that produce structures with no tubulin binding capacity (Borisy *et al.*, 1972; Capraro and Brossi, 1984). Irradiation of colchicine with UV light is known to cause a rearrangement of the C-ring to give lumicolchicine, which does not bind to tubulin (Wildman and Pursey, 1968; Borisey *et al.*, 1972). Analogous reactivity of the thiocolchicine chromophore would prevent its use as a photoaffinity analog. To minimize the likelihood of C ring rearrangement during

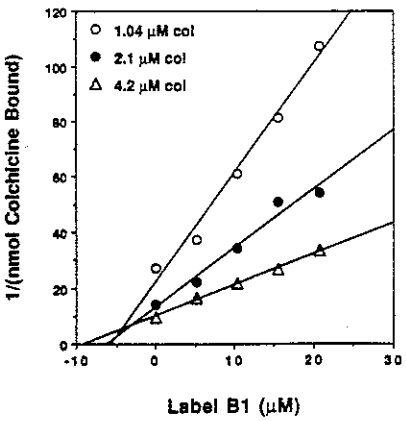


Figure 1. Competitive inhibition of colchicine and label **B1**. This modified Dixon plot demonstrates the effect of label **B1** on colchicine binding to tubulin. The concentration of label **B1** is plotted against the reciprocal of bound ^{3}H–colchicine at several concentrations of ^{3}H–colchicine. The data indicate competitive inhibition of label **B1** and colchicine with K_i of 4–5 μM.

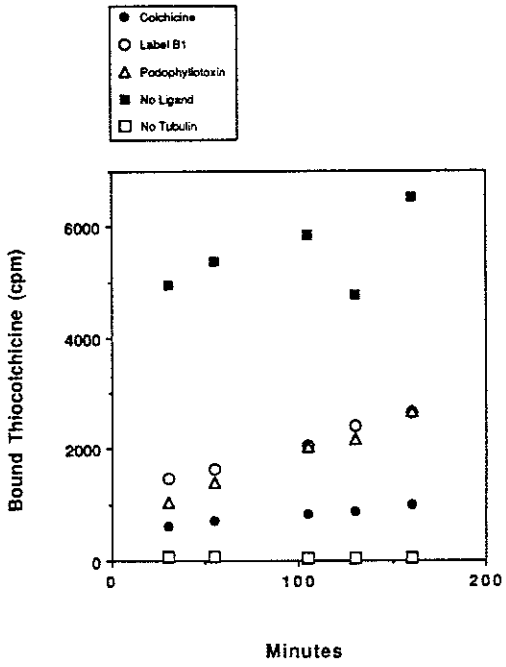


Figure 2. Reversibility of label **B1**–tubulin interaction. In order to compare the off rate of tubulin ligands, tubulin–ligand complex was incubated with excess ^{3}H–thiocolchicine. The thiocolchicine's displacement of bound ligand was monitored over time.

irradiation of the labels, reaction mixtures were irradiated through a solution filter of 2,7-dimethyl-3,6-diazacyclohepta-2,6-diene. This solution transmits primarily between 200 and 280 nm (Murov, 1973). When thiocolchicine was irradiated using the solution filter it was recovered unchanged.

Irradiation of **B1** and the allocolchicine label in methanol produced diastereomeric solvent capture products. Label **B1** gave no trace of C ring rearrangement (see Fig. 4). Thus the C ring

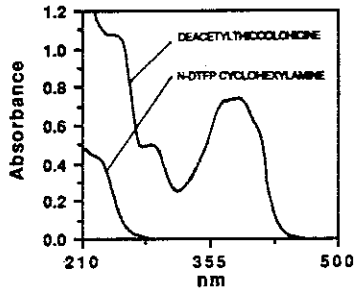


Figure 3. Ultraviolet spectra of *N*-DTFP cyclohexylamine and *N*-deacetylthiocolchicine. Both compounds are shown at 5×10^{-5} *M* in 95% ethanol.

rearrangement does not occur with thiocolchicine derivatives under the conditions used. The products were isolated and their structures determined on the basis of proton NMR and mass spectral analysis. The reaction of 0.05 mg/mL **B1** was complete in less than 10 min, as indicated by thin layer chromatography.

Label **B1** exhibited a pronounced reactivity in basic media such as aqueous ammonium carbonate/bicarbonate buffer at pH 8, or triethylamine in organic solvents. The starting material was transformed to a major product which was much more polar, as measured by TLC. The new compound was produced preparatively using triethylamine, and was isolated and purified by chromatography. Its FAB mass spectrum showed a molecular ion corresponding to the molecular weight of the starting material, and its proton NMR spectrum was indistinguishable from that of starting material, except for changes in the splitting pattern of the hydrogen α to the DTFP group. The product's IR spectrum showed no diazo absorbance, and its UV spectrum showed changes in the low wavelength absorptions stemming from the A ring and DTFP chromo-

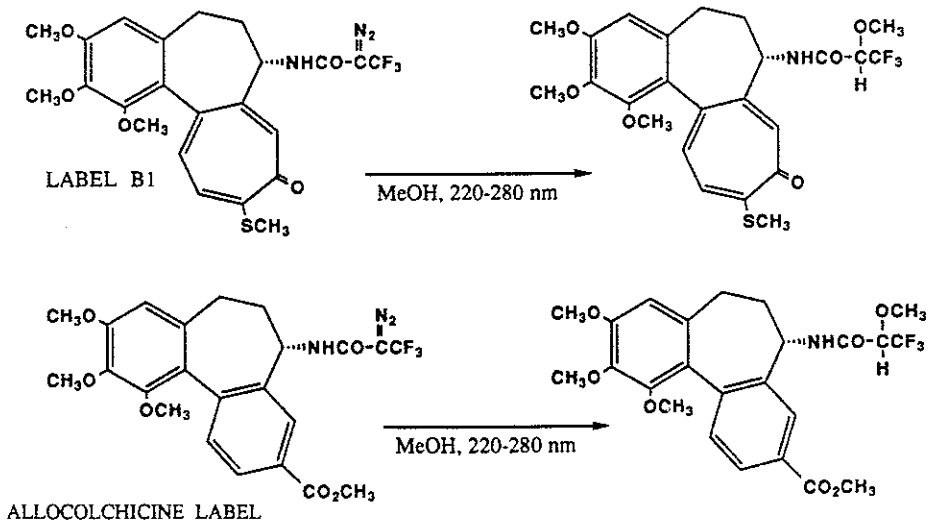


Figure 4. Photolytic solvent insertion reactions of label **B1** and allocolchicine label.

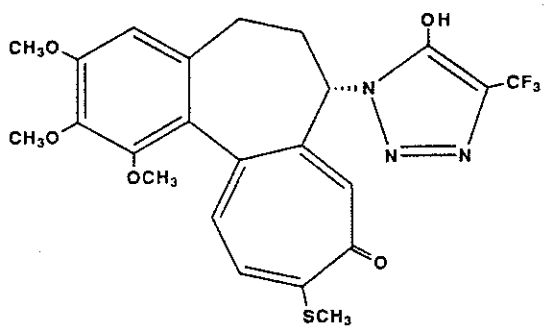


Figure 5. Product of label **B1** rearrangement.

phores. The structure **B1-R** (see Fig. 5) was assigned on the basis of these spectroscopic data and several precedents in which α-diazo carboxamides have been shown to reversibly cyclize to 1,2,3-triazolin-4-ones (Regitz and Anschutz, 1969; Buu and Edward, 1972; Franich *et al.*, 1972; Bartlett *et al.*, 1982; Oleson *et al.*, 1984).

In order to demonstrate that the reaction conditions would in fact produce a rearrangement of the *N*-DTFP group, (*N*-DTFP)cyclohexylamine was dissolved in dry dichloromethane with triethylamine. Although the reaction was much slower than with **B1**, a single product of much lower R_f was formed. The IR spectrum of this product no longer contained a diazo absorption, its UV spectrum showed the development of a new maximum at 250 nm, and its mass spectrum indicated that it had the same mass as starting material. When the α-diazoester labels **B2** and **A2** were subjected to the conditions giving **B1** rearrangement, no rearrangement was observed. The implications of this reaction for use of DTFP labels in photoaffinity experiments are discussed below.

Photolabeling studies

Complexes of tubulin with **B1** and **B2** were prepared and subjected to initial photolytic studies. Tubulin was incubated with 1 equiv of label at 37°C for 1 h. The tubulin-label complex was then separated from unbound label using the rapid microcolumn centrifugation technique of Penefsky (1979). Aliquots were analyzed to determine the fractional occupancy of tubulin, and photolysis was then carried out at <12°C for 30 min. The protein was then dialyzed against dilute ammonium bicarbonate, after which the level of incorporation of non-dialyzable radioactivity was determined.

Results of these experiments are summarized in Table 3. Label concentrations were determined by measuring radioactivity, and protein concentrations were determined using a modified Bradford assay (Bradford, 1976; Bane, 1983). Control experiments in which 1 equiv of colchicine was incubated with tubulin prior to label addition show substantially reduced labeling, indicating primarily specific binding to the colchicine site during complex formation. The achievement of substantial covalent attachment was confirmed by association of radioactivity with protein following both dialysis and HPLC fractionation. Control experiments in which irradiation was omitted exhibited some non-photochemical covalent incorporation, especially with **B2**.

Trypsin digests of the dialyzed protein were fractionated on C-18 HPLC columns under conditions similar to those developed by Postingl *et al.* (1981) for tubulin sequencing studies. While the radioactivity was concentrated in the later eluting peptides, the distribution was quite diffuse, indicating substantial non-specific labeling. The total radioactivity calculated from the HPLC is shown in column 6 of Table 3. HPLC traces and radioactivity profiles are shown in Figs. 6 and 7.

Table 3. Complex formation and covalent bonding

Label	Tubulin concentration	Equiv incubated	Equiv bound in complex	Equiv bound after photolysis + dialysis	Equiv bound after HPLC
B1	8.6	1	0.95	0.2	0.15
B1 (dark)	8.3	1	0.83	0.04	0.04
B2	8.8	1	1.1	0.3	0.25
B2	8.9	4.9	2.6	0.6	0.45
B2 (dark)	6.0	1	1.4	0.1	—
B2 (dark)	8.2	4.9	2.5	0.17	0.15
B2 (colchicine)	5.6	1	0.3	0.1	—
B2 (colchicine)	8.5	4.9	1.6	0.5	0.4

Tubulin concentration = mg/mL tubulin in irradiated solution. Equiv bound in complex = mol ligand bound per mol tubulin, prior to irradiation. Equiv bound after photolysis and dialysis = mol ligand bound covalently per mol tubulin, after irradiation of the non-covalent complex and dialysis to remove non-covalently attached ligand. Equiv bound after HPLC = mol ligand bound covalently per mol tubulin, determined by counting total radioactivity in tubulin tryptic digest products separated by HPLC. "Dark" control experiment = tubulin-label complex treated as in other experiments, but with irradiation omitted. "Colchicine" control experiments = tubulin–colchicine complex, rather than free tubulin, used in the experiment.

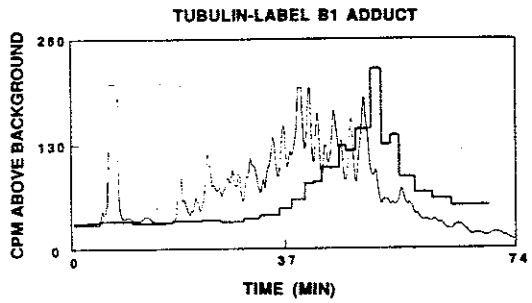


Figure 6. Tubulin-label adducts tryptic cleavage products—HPLC fractionation and radioactivity. Tryptic digests of the covalent tubulin-label adducts were fractionated by HPLC using gradients of increasing acetonitrile in aqueous ammonium bicarbonate buffer (for tubulin-**B1**), or aqueous trifluoroacetic acid (for tubulin-**B2**). The 215 nm absorbance of the HPLC eluant is shown superimposed on bar graphs indicating the radioactivity of fraction aliquots.

DISCUSSION

The photoaffinity analogs in Table 1 have been characterized structurally, and their effectiveness as tubulin assembly inhibitors has been determined. Strong inhibitory activity was taken as an indication that a DTFP analog showed strong binding to the normal colchicine–tubulin binding site, and thus fulfilled the first requirement of a useful photoaffinity analog. These experiments also shed light on the structural requirements for inhibitory activity.

Brossi and coworkers found that thiocolchicine is equipotent to colchicine in inhibition of ^{3}H–colchicine binding to tubulin (Rossner *et al.*, 1981). *N*-acyl analogs of thiocolchicine, *e.g.* benzoyl, trifluoroacetyl, and ethoxycarbonyl are at least as potent as thiocolchicine itself (Andreu *et al.*, 1984; Muzaffar *et al.*, 1990), and previous studies have demonstrated that a wide variety of substitutents can be introduced at the C-7 (nitrogen) position of colchicine. Our results show that a good level of activity is retained for both **B1**, in which the DTFP group is attached directly to the C-7 nitrogen, and in **B2**, in which the glycolic acid spacer is present.

There has been less study of substitution for the 1,2, and 3-methoxy positions of ring A. Using the competitive binding assay, Brossi and coworkers found the 2-demethyl and 3-demethyl derivatives of colchicine to be somewhat less strongly bound to tubulin than colchicine. Under conditions where colchicine produced 90% inhibition of ^{3}H–colchicine binding these compounds produced 50 and 68% inhibition, respectively. The 2-acetoxy analog produced 78% inhibition. Our results show the **A2** and **A3** DTFP derivatives to be much weaker inhibitors of tubulin polymerization than colchicine itself. This indicates weak tubulin binding, or lack of specific binding to the colchicine site, making these labels poor candidates for photoaffinity studies. The **AB-bis** label and allo label are slightly less tightly bound than labels **A2** and **A3**. The C ring label is the weakest inhibitor of all the analogs prepared.

Potent tubulin assembly inhibition by **B1** led to its selection for more detailed characterization. Competitive inhibition of colchicine binding by **B1** strongly suggested that it bound to the colchicine site. Label **B1** did not display the essentially irreversible tubulin binding found with colchicine, but instead was comparable to podophyllotoxin in the rate at which it was displaced from tubulin by ^{3}H–thiocolchicine. This reversible tubulin binding is a disadvantage for **B1** as a photoaffinity label. A label with the irreversible binding of colchicine would have enabled separation of tubulin-label complex from unbound label prior to irradiation, which should result in decreased non-specific tubulin labeling. Reversible binding by **B1** is, however, significant in revealing that relatively small differences between the colchicine and **B1** structures have produced profound effects on binding kinetics. The relationship between colchinoid structure and binding kinetics continues to be an important probe of drug–tubulin binding interactions (Garland, 1978; Ray *et al.*, 1981; Bane *et al.*, 1984; Engelborghs and Fitzgerald, 1987; Chabin and Hastie, 1989; Hastie, 1989).

The trypsin digestion is expected to cleave α- and β-tubulin at the basic amino acids arginine and lysine, generating a total of about 80 peptides ranging in molecular weight from about 300 for dipeptides to 6000. The HPLC analysis of the typical digested protein showed a large number of overlapping peaks, as expected. Aliquots of all eluted fractions were scintillation counted in order to compare the radioactivity profile with the absorbance profile

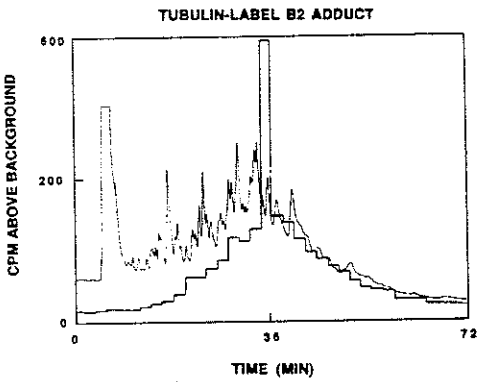


Figure 7. Tubulin-label adducts tryptic cleavage products—HPLC fractionation and radioactivity. Tryptic digests of the covalent tubulin-label adducts were fractionated by HPLC using gradients of increasing acetonitrile in aqueous ammonium bicarbonate buffer (for tubulin-**B1**), or aqueous trifluoroacetic acid (for tubulin-**B2**). The 215 nm absorbance of the HPLC eluant is shown superimposed on bar graphs indicating the radioactivity of fraction aliquots.

of the HPLC eluate. The radioactivity profile was displaced from the absorbance profile, with the highest levels of radioactivity occuring with slower eluting peptides. Nevertheless, the radioactivity profile was quite diffuse, indicating either the presence of numerous labeled peptides or poor resolution of covalently labelled material. This pattern was exhibited by digests from both the **B1** and **B2** labeled samples. Several of the fractions with the highest level of radioactivity were examined by the mass spectrometric method of Hunt and coworkers (1986, 1987). While several peptides which match the known sequence of porcine tubulin were found, no covalently labeled peptides could be identified. It appears that under the conditions of photoaffinity labeling used with **B1** and **B2** there is extensive non-specific labeling. There might be several causes, including non-specific chemical labeling, non-specific binding, or substantial photolysis of unbound label.

We believe our results reveal significant features of the DTFP group which bear on its use in constructing photoaffinity labels, especially when it is attached to the substrate as a secondary amide. Most obvious is the possibility of cyclization to the isomeric triazolinone structure. This transformation has literature precedent (Regitz and Anschutz, 1969; Buu and Edward, 1972; Franich *et al.*, 1972; Bartlett *et al.*, 1982; Oleson *et al.*, 1984) in cyclization of other α-diazocarboxamides. Recently an example of cyclization of an α-diazotrifluoropropanoyl amide was reported (Theodore *et al.*, 1990). A second feature is the poor separation of the DTFP amide chromophore from protein absorbance. The distinct maximum reported by Chowdry *et al.* (1976) at 255 nm is evidently characteristic of the DTFP-thio ester structure. We find that secondary aliphatic DTFP amides show a maxima at 216 nm with a shoulder at 240 nm.

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