Synthesis, Photochemical Reactions, and Tubulin Binding of Novel Photoaffinity Labeling Derivatives of Colchicine[1]

Marianne E. Staretz and Susan Bane Hastie*

Department of Chemistry, State University of New York at Binghamton, Binghamton, New York 13902-6000

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Introduction

Colchicine, an alkaloid from *Colchicum autumnale*, inhibits microtubule assembly by binding to a single high affinity site on the tubulin heterodimer.[2] The colchicine binding site on tubulin is a receptor site for a variety of antimitotic drugs, including podophyllotoxin, steganacin, combretastatin, and benzimidazole carbamates such as nocodazole. Colchicine binding is a ubiquitous property of mammalian tubulin, leading to the hypothesis that the colchicine binding site may be a receptor site for an unidentified endogenous regulator of microtubule dynamics.[3]

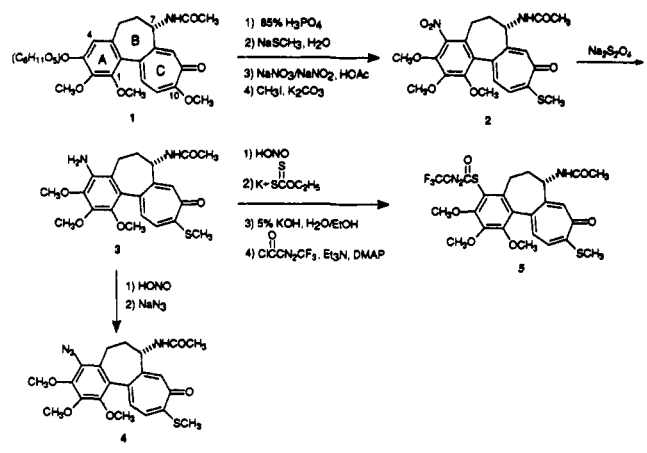
Attempts to identify the colchicine binding site on tubulin by affinity and photoaffinity labeling methods have provided conflicting results (for a recent review, see ref 2c). We have been engaged in the synthesis of novel photoaffinity labeling derivatives of colchicine designed to resolve the ambiguities from the previous efforts.[4,5] In this work, the synthesis, tubulin binding properties, and photochemical decomposition of two novel colchicine-based photoaffinity labels are described. The labels were designed to retain high affinity for the colchicine site on tubulin while positioning the photolabile groups on a position of the parent molecule predicted to be at or near the interior of the binding site.

Results

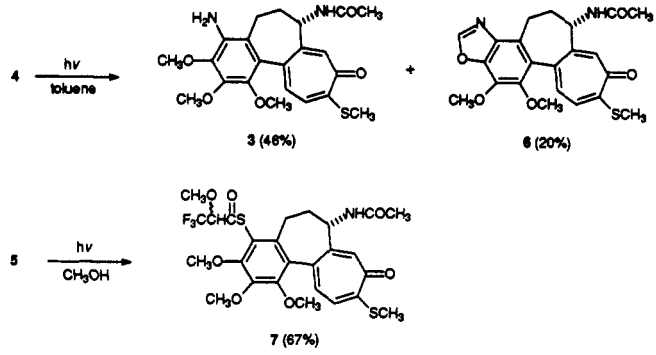
Synthesis. The two A ring photoaffinity labels (**4** and **5**) were prepared from a common intermediate (**3**) as shown in Scheme I. Thiocolchicine rather than colchicine was selected as the parent ligand due to the greater photochemical stability of the tropone ring in this molecule. We have previously shown that the tubulin binding properties of colchicine and thiocolchicine are virtually identical.[6]

Compound **2** was obtained from colchicoside (**1**) in an overall yield of 46%. This route allows for ready inclusion of a radioactive isotope in the photoaffinity labels. Colchicoside is no longer commercially available, but **2** may also be prepared directly from thiocolchicine, in low yield, by reaction with $NaNO_2$ in CF_3COOH.

Scheme I



Scheme II



Label **4** was prepared from **3** by reaction of the diazonium salt of **3** with NaN_3. The carbene precursor of label **5** was attached to the thiocolchicinoid skeleton through a thioester linkage rather than an amide linkage due to the superior stability of the thioester. N-Acylated diazo compounds have been shown to rearrange to triazolinone structures in solution.[5,7]

Photolyses. As an aid to estimating the effectiveness of the colchicinoids as photoaffinity labels, the compounds were photolyzed in solvent and the resulting major products identified (Scheme II). Photolysis of **4** in methanol and in toluene resulted in the formation of **3**. Upon photolysis in toluene, a second product (**6**) from intramolecular reaction of the nitrene was also isolated. Compound **6** is presumably formed from hydrogen abstraction from the C-3 methoxy group by the nitrene. The resulting biradical may then collapse to form a benzoxazoline ring, oxidation of which would lead to the observed product. Insertion into the C–H bond of a substituent ortho to the source of the nitrene has been observed previously, with attack at the β-position being greatly preferred.[8]

The major product (**7**) from the photolysis of **5** in methanol was due to insertion of the carbene into the solvent. A few additional, minor products were observed by TLC, but isolation and characterization of these products was not possible due to the small scale of the reaction. Photolysis of **5** in toluene led to a tarry mixture from which no products were characterized.

(1) This work was supported by the National Science Foundation (DMB 90-05614).

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Table I. Inhibition of in Vitro Microtubule Assembly and [^{3}H]Colchicine Binding to Tubulin by 4 and 5

compound	I_{50}, μM[a]	K_I, μM[b]
4	2.5	1.6
5	25	76
colchicine	5.1	4.2

[a] I_{50} is the concentration of ligand required to inhibit in vitro microtubule assembly by 50% relative to a control without added ligand. [b] K_I is the competitive inhibition constant for competition of the ligand with [^{3}H]colchicine binding to tubulin, obtained as described in ref 13.

Biochemical Analyses. The tubulin binding properties of 4 and 5 were indirectly assessed by evaluating their ability to inhibit in vitro microtubule assembly (Table I). Label 4 was found to be more effective than colchicine at inhibiting microtubule assembly, while label 5 was less effective but still reasonably potent. To demonstrate that the inhibition of microtubule assembly is due to binding to tubulin at the colchicine site, the effect of the photoaffinity labels on the binding of [^{3}H]colchicine to tubulin was assessed. Lineweaver–Burk analyses of the data showed that both photoaffinity labels behaved as competitive inhibitors of colchicine binding for tubulin. The apparent inhibition constants (K_Is) are shown in Table I. Again, label 4 was found to be slightly more potent than colchicine while label 5 was less potent.

Discussion

Both of the novel photoaffinity labels prepared for the colchicine binding site on tubulin possess photochemical and tubulin binding properties suitable for identifying the colchicine binding site on tubulin. The nitrene precursor, 4, retains high affinity for the protein and appears to bind to the colchicine site with higher affinity than colchicine itself. Upon photolysis of 4 in toluene, about 20% of 6 was formed as a result of intramolecular C–H insertion. Intramolecular reactions may be more indicative of reactions that occur during the photoaffinity labeling experiment than intermolecular reactions, as the photoaffinity labeling reagent is essentially immobilized in the ligand binding site.[9] Thus, the presence of the intramolecular reaction product from 4 indicates its possible effectiveness as a photoaffinity label for tubulin.

The carbene precursor, 5, appears to have decreased affinity for the colchicine site on tubulin relative to 4 and colchicine. These results indicate that there is a structural requirement at the C-4 position for high affinity binding to tubulin, although the nature of this requirement is unclear from the small number of C-4 derivatives of colchicine that have been prepared to date.[10] A decrease in affinity of the photoaffinity label for tubulin can be accommodated in the photoaffinity labeling experiment by increasing the concentration of the photoaffinity label to drive the equilibrium toward the complex. The lack of significant Wolff rearrangement of the carbene coupled with the observed formation of the solvent insertion product indicates that this label will also be suitable for the photoaffinity labeling experiment.

Experimental Procedures

Syntheses. General. General information about the instrumentation and chromatography used for synthesis is found in ref 4. Structures of known compounds were confirmed by NMR ($CDCl_3$ reference), IR, and LRMS. Colchicoside (1) was kindly supplied by Roussel-UCLAF laboratories in Paris, France.

3-Demethylcolchicine. 3-Demethylcolchicine was synthesized according to a literature procedure.[11] The literature procedure uses methylene chloride for extraction of the product. Chloroform was found to provide a more efficient extraction of the product. The yield was 79% and the compound was used without further purification.

3-Demethylthiocolchicine. This compound was prepared according to a literature procedure.[12] The product was purified by column chromatography (90:10 methylene chloride–methanol) and was obtained in a yield of 89%.

3-Demethyl-4-nitrothiocolchicine. This compound was prepared from 3-demethylthiocolchicine by nitrosation under oxidizing conditions. 3-Demethylthiocolchicine (900 mg, 2.25 mmol) was dissolved in a mixture of 3.0 mL of acetic acid and 3.0 mL of water. Powdered sodium nitrate (235 mg, 2.8 mmol) followed by powdered sodium nitrite (235 mg, 3.4 mmol) was added, and the solution was stirred at room temperature for 30 min. The mixture was then diluted with water and extracted three times with chloroform. The organic extracts were combined and extracted three times with water. The organic layer was dried over sodium sulfate and evaporated to yield a brown solid. In order to fully characterize this novel product, it was purified by column chromatography (90:10 ethyl acetate–methanol). When complete characterization was not required, the product was methylated without any further purification as the nitrated demethylated product decomposes quite readily. The purified product was brownish orange in color and was obtained in a yield of 65%. **3-Demethyl-4-nitrothiocolchicine**: mp 198–200 °C; $[\alpha]^{25}_D$ −265° (*c* 1.0); ^{1}H NMR δ 7.61 (br, 1 H, NH), 7.42 (s, 1 H, H-8), 7.19 (d, 1 H, H-12, J = 10.2 Hz), 7.07 (d, 1 H, H-11, J = 10.5 Hz), 4.66 (p, 1 H, H-7), 4.01 (s, 3 H, 2-OCH_3), 3.70 (s, 3 H, 1-OCH_3), 2.88 (m, 1 H, H-5), 2.44 (s and m, 4 H, SCH_3 and H-5), 2.21 (m, 1 H, H-6), 2.03 (s, 3 H, $COCH_3$), 1.89 (m, 1 H, H-6); ^{13}C NMR δ 182.3, 170.3, 159.8, 153.5, 150.7, 145.9, 140.4, 136.1, 135.1, 133.7, 128.7, 128.3, 126.2, 126.0, 61.64, 61.61, 52.1, 35.1, 25.5, 22.8, 15.2; IR 3510–3260 (br), 3450, 3320, 1672, 1605 cm^{-1}; LRMS (*m*/*z*) molecular ion peak not observed, 402 (M − O − CO), 401 (M − OH − CO), 377, 314; HRMS calcd for $C_{20}H_{22}O_5N_2S_1$ (M − O, M − CO) 402.1248; found 402.1262.

4-Nitrothiocolchicine (2). 3-Demethyl-4-nitrothiocolchicine (500 mg, 1.12 mmol) in 5 mL of acetone containing 1.5 g of potassium carbonate was treated with 3 equiv of methyl iodide. The solution was stirred for 18 h at room temperature. The reaction mixture was filtered to remove the solid potassium carbonate and the acetone was evaporated. The residue was dissolved in methylene chloride, dried over sodium sulfate, filtered, and evaporated to yield an orange-brown solid. The solid was purified by column chromatography (90:10 ethyl acetate–methanol) and obtained in a 66% yield. Compound 2: mp 157–159 °C; $[\alpha]^{25}_D$ −123° (*c* 1.0); ^{1}H NMR δ 8.01 (br, 1 H, NH), 7.43 (s, 1 H, H-8), 7.22 (d, 1 H, H-12, J = 10.5 Hz), 7.07 (d, 1 H, H-11, J = 10.5 Hz), 4.64 (p, 1 H, H-7), 4.02 (s, 3 H, 3-OCH_3), 3.99 (s, 3 H, 2-OCH_3), 3.68 (s, 3 H, 1-OCH_3), 2.54 (m, 1-H, H-5), 2.44 (s, 3 H, SCH_3), 2.29 (m, 1 H, H-5), 2.20 (m, 1 H, H-6), 2.01 (s, 3 H, $COCH_3$), 1.89 (m, 1 H, H-6); ^{13}C NMR δ 182.3, 170.3, 160.0, 152.8, 150.7, 146.1, 145.5, 141.2, 135.9, 135.0, 129.4, 128.4, 126.2, 125.8, 62.3, 61.7, 61.5, 52.0, 35.0, 24.7, 22.8, 15.1; IR 3420, 3300–3250, 3020–2850, 1660, 1600, 1525, 1343; LRMS (*m*/*z*) 460 (M^+), 462 (M + 2), 432, 430, 427; HRMS calcd for $C_{22}H_{24}O_7N_2S$ 460.1303, found 460.1272.

4-Aminothiocolchicine (3). Compound 2 (0.250 g, 0.54 mmol) was dissolved in a mixture of water (5 mL) and methanol (1.5 mL) containing 5% potassium carbonate. To this mixture was added small portions of sodium dithionite until the reaction appeared complete by TLC (90:10 ethyl acetate–methanol). Upon completion of the reaction, the solution was diluted with 10 mL of water and was extracted five times with 20-mL portions of

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chloroform. The organic extracts were combined, dried over sodium sulfate, and evaporated to yield an orange solid. The product was purified by radial chromatography (90:10 ethyl acetate–methanol) and was obtained in a yield of 58%. Compound 3: mp 167–168 °C; $[\alpha]^{25}_D$ −117° (c 1.0); UV(EtOH) 216 nm ($\epsilon = 2.52 \times 10^4$ M^{-1} cm^{-1}), 368 nm ($\epsilon = 1.24 \times 10^4$ M^{-1} cm^{-1}); ^{1}H NMR δ 7.80 (br, 1 H, NH), 7.42 (s, 1 H, H-8), 7.31 (d, 1 H, H-12, J = 10.2 Hz), 7.08 (d, 1 H, H-11, J = 10.5 Hz), 4.65 (p, 1 H, H-7), 3.97 (s, 3 H, 3-OCH_3), 3.93 (s, 3 H, 2-OCH_3), 3.55 (s, 3 H, 1-OCH_3), 2.62 (dd, 1 H, H-5), 2.43 (s, 3 H, SCH_3), 2.24 (m, 1 H, H-5), 2.10 (m, 1 H, H-6), 1.99 (s, 3 H, $COCH_3$), 1.83 (m, 1 H, H-6); ^{13}C NMR δ 182.4, 170.0, 158.3, 151.6, 145.5, 142.9, 141.4, 138.6, 134.9, 133.0, 129.2, 128.2, 126.7, 118.0, 61.7, 61.2, 60.5, 52.4, 35.0, 22.9, 22.8, 15.1; IR 3420, 3380–3220 (br), 3020–2820, 1660, 1600, 1352 cm^{-1}; LRMS (m/z) 430 (M^+), 432 (M + 2), 402, 328; HRMS calcd for $C_{22}H_{26}O_5N_2S$ 430.1561, found 430.1550.

4-Azidothiocolchicine (4). Compound 3 (20 mg, 0.047 mmol) was dissolved in 1 mL of water and 0.4 mL of concentrated HCl and the solution was cooled on ice. Sodium nitrite (0.018 g, 0.261 mmol) was dissolved in 5 mL of water and 1 mL of this solution (0.052 mmol) was added to the reaction mixture. The reaction was allowed to stir on ice for 1 h. Meanwhile, a solution of NaN_3 (0.017 g in 5 mL of water) was prepared and 1 mL of this solution was added to the reaction mixture, which was then stirred for an additional 1 h. The mixture was diluted with 10 mL of water and extracted three times with chloroform. The product was purified by radial chromatography (in dim incandescent light) using 50:50 acetone–petroleum ether. The yield of 4, which was a yellow solid, was 72%. Compound 4: mp 118–120 °C; $[\alpha]^{25}_D$ −96° (c 1.0); UV (EtOH) 256 nm ($\epsilon = 2.30 \times 10^4$ M^{-1} cm^{-1}, 364 nm ($\epsilon = 1.36 \times 10^4$ M^{-1} cm^{-1}); ^{1}H NMR δ 7.85 (d, 1 H, NH), 7.39 (s, 1 H, H-8), 7.23 (d, 1 H, H-12, J = 10.6 Hz), 7.05 (d, 1 H, H-11, J = 10.5 Hz), 4.54 (p, 1 H, H-7), 3.99 (s, 3 H, 3-OCH_3), 3.96 (s, 3 H, 2-OCH_3), 3.59 (s, 3 H, 1-OCH_3), 3.06 (dd, 1 H, H-5), 2.42 (s, 3 H, SCH_3), 2.17 (m, 1 H, H-5), 1.98 (s, 3 H, $COCH_3$), 1.93 (m, 1 H, H-6), 1.79 (m, 1 H, H-6); ^{13}C NMR δ 182.4, 170.0, 159.0, 151.3, 148.2, 148.1, 146.0, 137.5, 134.8, 129.6, 128.2, 126.4, 126.1, 126.0, 61.8, 61.7, 61.4, 52.2, 35.2, 29.8, 22.8, 15.1; IR 3460, 3300, 3040–2850, 2118, 1670, 1620 cm^{-1}; LRMS (m/z) M^+ not observed, 426 (100). Attempts to obtain HRMS of 4 were unsuccessful.

4-Mercaptothiocolchicine. Compound 3 (40 mg, 0.093 mmol) was dissolved in 1 mL of water containing 0.1 mL of concentrated HCl and the solution was cooled on ice. Sodium nitrite (0.036 gm) was dissolved in 5 mL of water and 1 mL of this solution was added to the reaction mixture. The solution was allowed to stir on ice for 1 h. In the meantime, a solution of *o*-ethylxanthic acid, potassium salt (0.018 g, 0.11 mmol) in 3 mL of water was prepared and heated to 40 °C. The solution of the diazonium salt was added *slowly* to the solution of *o*-ethylxanthic acid in water (at 40 °C). After the addition was complete, the solution was stirred for 20 min at 40 °C. This solution was then extracted three times with methylene chloride. The organic extracts were combined, dried over sodium sulfate, and evaporated to yield a light yellow solid. This solid was dissolved in a solution of 3 mL of water and 2 mL of ethanol containing 5% NaOH. The solution was stirred at room temperature until TLC showed complete hydrolysis of the xanthate ester. Using TLC conditions of 95:5 ethyl acetate–methanol, the R_f values of the product and starting material were 0.41 and 0.47, respectively. The reaction mixture was acidified and extracted three times with chloroform. The organic extracts were combined, dried over sodium sulfate, and evaporated to yield a yellow solid. This solid was purified by radial chromatography (95:5 ethyl acetate–methanol) and was obtained in a yield of 65%: mp 166–167 °C; $[\alpha]^{25}_D$ −101° (c 1.0); ^{1}H NMR δ 7.80 (d, 1 H, NH), 7.44 (s, 1 H, H-8), 7.28 (d, 1 H, H-12, J = 10.2 Hz), 7.09 (d, 1 H, H-11, J = 10.5 Hz), 4.61 (p, 1 H, H-7), 4.28 (s, 1 H, SH), 3.98 (s, 6 H, 3-OCH_3 and 2-OCH_3), 3.61 (s, 3 H, 1-OCH_3), 2.91 (m, 1 H, H-5), 2.45 (s, 3 H, SCH_3), 2.27 (m, 2 H, H-5 and H-6), 2.01 (s, 3 H, $COCH_3$), 1.85 (m, 1 H, H-6); ^{13}C NMR δ 182.4, 170.0, 158.8, 151.3, 148.9, 148.5, 145.9, 137.7, 134.8, 130.6, 130.0, 128.2, 126.4, 120.3, 61.6, 61.3, 60.3, 52.3, 34.5, 27.1, 22.8, 15.1; IR 3458, 3300, 3040-2850, 2580 (w), 1675, 1610; LRMS (m/z) 447 (M^+), 449 (M + 2), 419, 432; HRMS calcd for $C_{22}H_{25}O_5NS_2$ 447.1173, found 447.1142.

4-[(2-Diazo-3,3,3-trifluoropropanoyl)thio]thiocolchicine (5). A solution of 4-mercaptothiocolchicine (30 mg, 0.067 mmol), triethylamine (19 μL, 2 equiv), and 4-(dimethylamino)-pyridine (1.6 mg, 0.20 equiv) in 2 mL of dry methylene chloride was treated *slowly* with 1.5 equiv of 2-diazo-3,3,3-trifluoropropanoyl chloride. The mixture was allowed to stir for 15 min at room temperature. The reaction mixture was then extracted with a 5% HCl solution. The organic layer was dried over sodium sulfate and evaporated to yield a light yellow solid. The product was purified by radial chromatography (95:5 ethyl acetate–methanol) and was obtained in a yield of 91%. Compound 5: mp 181–182 °C; $[\alpha]^{25}_D$ −102° (c 1.0); UV (EtOH) 256 nm ($\epsilon = 2.30 \times 10^4$ M^{-1} cm^{-1}), 364 nm ($\epsilon = 1.36 \times 10^4$ M^{-1} cm^{-1}); ^{1}H NMR δ 7.80 (d, 1 H, NH), 7.41 (s, 1 H, H-8), 7.28 (d, 1 H, H-12, J = 10.6 Hz), 7.10 (d, 1 H, H-11, J = 10.4 Hz), 4.58 (p, 1 H, H-7), 3.98 (s, 3 H, 3-OCH_3), 3.94 (s, 3 H, 2-OCH_3), 3.70 (s, 3 H, 1-OCH_3), 3.17 (m, 1 H, H-5), 2.46 (s, 3 H, SCH_3), 2.20 (m, 2 H, H-5 and H-6), 2.00 (s, 3 H, $COCH_3$), 1.86 (m, 1 H, H-6); ^{13}C NMR δ 182.4, 178.1, 169.9, 159.2, 155.5, 153.9, 150.6, 146.2, 139.1, 137.2, 134.8, 130.0, 128.0, 126.2, 124.0, 112.8, 61.6, 61.5, 61.4, 52.2, 35.4, 27.4, 27.2, 22.9, 15.2; IR 3420, 3250, 3020–2850, 2105, 1660, 1600 cm^{-1}; LRMS, molecular ion not observed. Attempts to obtain HRMS of 5 were unsuccessful.

Alternative Procedure for the Preparation of 2. Compound 2 was also synthesized directly from thiocolchicine, albeit in low yield. Thiocolchicine (60 mg, 1.4 mmol) was dissolved in 2 mL of trifluoroacetic acid. A solution of sodium nitrite (2 equiv) in 2 mL of trifluoroacetic acid was added slowly to the solution of thiocolchicine. The reaction mixture was stirred for 30 min, after which it was diluted with water, neutralized with 5 N NaOH, and extracted three times with chloroform. The organic extracts were combined, dried over sodium sulfate, and evaporated to yield 2 in 21% yield.

Photolyses. General. Compounds were photolyzed using the following general procedure. The compound was dissolved in solvent at a concentration of 1 mg mL^{-1}. The solution was purged with nitrogen and was irradiated in quartz using the short wavelength lamp (254 nm) of a Spectroline (Model ENF-24) ultraviolet lamp. The photolysis was monitored by UV–visible spectroscopy and was continued until no further change in the UV–visible spectrum was observed. When photolysis was complete, the solvent was evaporated.

Photolysis of Compound 4 in Toluene. Compound 4 in toluene was photolyzed as described above except that the long wavelength lamp (350 nm) was used to irradiate the solution. TLC of the photolyzed sample showed the presence of two spots with R_f values of 0.48 and 0.23, respectively (95:5 ethyl acetate–methanol). The spot with the lower R_f was isolated and was found to be 3. The spot with the higher R_f value (6) is apparently due to an intramolecular reaction. The yields of 3 and 6 were 46% and 20%, respectively. **3,4-Oxazole fused 3-demethoxythiocolchicine 6**: UV (EtOH) 216, 292 (sh), 256, 370 nm; ^{1}H NMR δ 8.01 (s, 1 H, -OC*H*N-), 7.33 (d, 1 H, H-12), 7.28 (s, 1 H, H-8), 7.08 (d, 1 H, H-11), 6.43 (d, 1 H, NH), 4.62 (p, 1 H, H-7), 4.28 (s, 3 H, 2-OCH_3), 3.66 (s, 3 H, 1-OCH_3), 3.46 (m, 1 H, H-5), 2.48 (s, 3 H, SCH_3), 2.63 (m, 2 H, H-5 and H-6), 2.02 (s, 3 H, $COCH_3$), 1.90 (m, 1 H, H-6); ^{13}C NMR δ 181.9, 169.1, 159.1, 151.8, 150.0, 146.3, 145.2, 137.2, 137.0, 135.2, 134.8, 130.3, 128.0, 125.8, 123.4, 61.4, 40.5, 35.7, 29.2, 22.3, 14.7; IR 3450, 3300, 3200–2850, 1675, 1608 cm^{-1}; LRMS (m/z) 426 (M^+), 393, 367, 339, 327, 324, 308; HRMS calcd for $C_{22}H_{22}N_2O_5S$ 426.1249, found 426.1270.

Photolysis of Compound 4 in Methanol. TLC and spectral analysis revealed that the major product of the reaction was 3. The product was obtained in a yield of 74%.

Photolysis of Compound 5 in Methanol. The major product (7) was purified by radial chromatography using 95:5 ethyl acetate–methanol and was obtained as a yellow solid in a yield of 67%. Attempts were made to separate the diastereomers without success. The R_f of the diastereomers was 0.42 using 95:5 ethyl acetate–methanol and 0.47 using 95:5 methylene chloride–methanol. **4-[(2-Methoxy-3,3,3-trifluoropropanoyl)thio]thiocolchicine (7)**: UV (EtOH) 216, 260 (sh), 288 (sh), 366 nm; ^{1}H NMR δ 7.32 and 7.30 (s, 1 H, H-8), 7.24 and 7.21 (d, 1 H, H-12), 7.05 and 7.06 (d, 1 H, H-11), 7.07 and 6.95 (d, 1 H, NH), 4.54 (p, 1 H, H-7), 4.35 (m, 1 H, SCOC*H*OCH$_3$CH$_3$), 4.03 and 4.01 (s, 3 H, 3-OCH_3), 3.97 (s, 2-OCH_3), 3.84 and 3.79 (s, 3 H, SCOCHOC*H*$_3$-

CF_3), 3.65 and 3.64 (s, 3 H, 1-OCH_3), 3.57 (m, 1 H, H-5), 2.48 (s, 3 H, SCH_3), 2.15 (m, 2 H, H-5 and H-6), 2.01 (s, 3 H, $COCH_3$), 1.85 (m, 1 H, H-6). The product exists as a pair of distereomers. Therefore, some of the signals in the ^1H NMR spectrum are doubled. ^{13}C NMR δ 183.8, 182.3, 169.7, 159.3, 153.2, 149.9, 138.5, 137.0, 135.0, 134.6, 134.5, 130.0, 128.1, 126.0, 118.2, 103.0, 74.8, 61.5, 61.4, 61.1, 53.3 and 52.0, 35.8 and 35.5, 26.9 and 26.8, 23.1, 15.2; IR 3450, 3300, 3100–2850, 1675, 1620; LRMS (m/z) 587 (M^+), 559, 500, 387, 359, 327; HRMS calcd for $C_{26}H_{28}NO_7S_2F_3$ 587.1257, found 587.1230.

Biochemical Analyses. General. Sources of materials and procedures for routine preparation of tubulin are found in ref 4. The following extinction coefficients for the specified compounds in aqueous solution were used: colchicine, $\epsilon_{352} = 1.69 \times 10^4$ M^{-1} cm^{-1}; **4**, $\epsilon_{368} = 1.20 \times 10^4$ M^{-1} cm^{-1}; **5**, $\epsilon_{368} = 1.84 \times 10^4$ M^{-1} cm^{-1}. Microtubule assembly experiments were performed in PME buffer (PME buffer = 0.1 M Pipes, 1 mM $MgSO_4$, 2 mM EGTA, pH 6.90 at 23 °C). All other experiments were performed in PMEG buffer (PME buffer containing 0.1 mM GTP).

Inhibition of Microtubule Assembly. Microtubule protein at a concentration of 2 mg mL^{-1} was incubated with the appropriate ligand at the desired concentrations in PME buffer for 20 min at room temperature. GTP was then added to achieve a final concentration of 1 mM. Assembly was initiated by warming the solution to 37 °C and the polymerization process was monitored by observing the change in turbidity of the solution at 400 nm on a Beckman (Model 25) UV–visible spectrophotometer containing a thermostated cell holder. The I_{50} (ligand concentration required to produce a 50% inhibition of microtubule assembly relative to a control without added ligand) was determined by interpolation from a plot of percent inhibition vs ligand concentration. The percent inhibition was determined from the plateau absorbance values after polymerization of the microtubule protein.

Competitive Binding Assay. The ability of a ligand to competitively inhibit the binding of [^3H]colchicine to tubulin was assessed as previously described.[13]

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Supplementary Material Available: ^1H NMR spectra of compounds 2–7, 3-demethyl-4-nitrothiocolchicine, and 4-mercaptothiocolchicine (8 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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