Table II. Narcotic Agonist Activity of *N*-Methyl Analogues in Various Assays[a]

compd	M_r	6-subst	tail flick, mg/kg (95% CL)	PPQ writhing, mg/kg (95% CL)	hot plate, mg/kg (95% CL)	Nilsen, mg/kg (95% CL)
4c	397.3	NHNH	0.70 (0.30–1.7)	0.05 (0.01–0.20)	0.81 (0.67–0.97)	2.0 (0.4–2.9)
5c	315.4	=NOH	2.2 (1.4–3.6)	0.10 (0.03–0.40)	0.56 (0.40–0.77)	0.45 (0.31–0.65)
6c	348.4	=NOMe	0.08 (0.02–0.3)	0.02 (0.01–0.03)	0.22 (0.20–0.24)	not done
morphine sulfate			5.8 (5.68–5.92)	0.23 (0.204–0.256)	0.98 (0.83–1.1)	1.3 (1.0–19.7)
dihydromorphine hydrochloride					0.19 (0.15–1.25)	0.2 (0.15–0.30)

[a] Compounds were tested as previously described; see footnote *a* to Table I. All compounds were given subcutaneously as aqueous solutions of HCl salts.

silica gel (90 g, 70–325 mesh), eluting with EtOAc–EtOH–NH_3 (aqueous) (100:1:1). The gelatinous solid was dissolved in benzene and precipitated with petroleum ether (bp 30–60 °C), affording 1.06 g (34%) of oxime **5a** as a white solid. Alternatively, **5a** was prepared from equal to molar quantities of naloxone, NH_2OH·HCl, and aqueous 2 N NaOH in methanol at room temperature. Water was added and the oxime extracted into $CHCl_3$. Evaporation and crystallization afforded oxime **5a** in 90–95% yield: mp 118–119 °C; NMR ($CDCl_3$) δ 4.98 (s, C_5 H); $[\alpha]^{25}_D$ -270° (EtOH, *c* 1.0). Anal. **5a** ($C_{19}H_{22}N_2O_4$) C, H, N.

The diaziridine was eluted from the column using EtOAc–EtOH–NH_3 (aqueous) (100:10:1). Crystallization from EtOAc afforded 1.73 g (55%) of **4a** as a pale yellow solid: mp 159–160 °C; NMR ($CDCl_3$) δ 4.78 (s, C_5 H); $[\alpha]^{25}_D$ -149.6° (EtOH, *c* 1.0). Anal. **4a** ($C_{19}H_{23}N_3O_3$) C, H, N.

6,6-Hydrazi- and 6-Oximinonaltrexone (4b and 5b). The compounds were prepared by a procedure similar to that described for the preparation of **4a** and **5a**. From 3.0 g (8.8 mmol) of naltrexone (1b) and 1.13 g (9.7 mmol) of hydroxylamine-*O*-sulfonic acid was obtained 1.05 g (34%) of naltrexone oxime (**5b**) [mp 235–236 °C (THF–petroleum ether); NMR ($CDCl_3$) δ 5.00 (s, C_5 H); $[\alpha]^{25}_D$ -330° (EtOH, *c* 1.0)] and 1.68 g (54%) of the 6,6-diaziridine of naltrexone (**4b**) [mp 198–199.5 °C (EtOAc); NMR ($CDCl_3$) δ 4.80 (s, C_5 H); $[\alpha]^{25}_D$ -169.3° (EtOH, *c* 1.0)]. Anal. **5b** ($C_{20}H_{24}N_2O_4$) C, H, N. Anal. **4b** ($C_{20}H_{25}N_3O_3$) C, H, N.

6,6-Hydrazi- and 6-Oximinooxymorphone (4c and 5c). These compounds were prepared by a procedure similar to that described for the preparation of **4a** and **5a**. From 3.5 g (11.6 mmol) of oxymorphone and 1.5 g (12.8 mmol) of hydroxylamine-*O*-sulfonic acid was isolated 1.32 g (36%) of oxymorphone oxime (**5c**) [mp 270–271 °C (THF); NMR (acetone-d_6) δ 4.83 (s, C_5 H); $[\alpha]^{25}_D$ -350° (EtOH, *c* 1.0)] and 2.00 g (55%) of 6,6-hydrazioxymorphone (**4c**) [mp 215–216 °C (EtOAc); NMR ($CDCl_3$) δ 4.78 (s, C_5 H); $[\alpha]^{25}_D$ -126.0° (EtOH, *c* 1.0)]. Anal. **5c** ($C_{17}H_{20}N_2O_4$) C, H, N. Anal. **4c** ($C_{17}H_{21}N_3O_3$) C, H, N.

6-Oximinonaloxone *O*-Methyl Ether (6a). Naloxone (**1a**) (1.00 g, 3.05 mmol) was added in portions to a solution of $MeONH_2$·HCl (0.330 g, 3.9 mmol) in a mixture of 1.6 mL of 10% aqueous NaOH and 15 mL of MeOH. The mixture was refluxed for 5 h, cooled, diluted with 50 mL of H_2O, and extracted with $CHCl_3$ (3 × 15 mL). The combined $CHCl_3$ extracts were dried (Na_2SO_4) and evaporated. The yellow oil obtained after evaporation of the solvent solidified on standing at room temperature was crystallized from hexane–petroleum ether (bp 35–60 °C), affording 1.03 g (95%) of the ketoxime *O*-methyl ether: mp 128–129 °C; NMR ($CDCl_3$) δ 4.95 (s, C_5 H), 3.82 (s, OCH_3); $[\alpha]^{25}_D$ -229.6° (MeOH, *c* 0.25). Anal. ($C_{20}H_{24}N_2O_4$) C, H, N.

In a similar way the ketoxime *O*-methyl ethers of naltrexone and oxymorphone (**6b** and **6c**) were prepared in 90–95% yield. 6-Oximinonaltrexone *O*-methyl ether (**6b**): mp 172–173 °C; NMR ($CDCl_3$) δ 4.95 (s, C_5 H), 3.82 (s, OCH_3); $[\alpha]^{25}_D$ -212.8° (MeOH), *c* 0.25). Anal. **6b** ($C_{21}H_{26}N_2O_4$) C, H, N. 6-Oximinooxymorphone *O*-methyl ether (**6c**): mp 115–116 °C; NMR ($CDCl_3$) δ 4.92 (s, C_5 H), 3.85 (s, OCH_3); $[\alpha]^{25}_D$ -175.0° (MeOH, *c* 0.25). Anal. **6c** ($C_{18}H_{22}N_2O_4 \cdot H_2O$) C, H, N.

Mutagenesis Testing. The mutagenesis assays in bacterial tester strains TA-98 and TA-100 using the 9000*g* supernatant fraction from livers of male rats treated with Arochlor 1254 were performed as previously described.[11]

Acknowledgment. We acknowledge support of this work by NIDA Research Grant DA-2370 and testing of compounds by the Committee on Problems of Drug Dependence, through Dr. Arthur Jacobson, NIADDK. We also thank Dr. Jacobson for providing us data on oxymorphone oxime, which was also prepared in his laboratory. We thank Dr. Peter J. Wirth, NCI, for testing the compounds in mutagenesis assays.

Registry No. **1a**, 465-65-6; **1b**, 16590-41-3; **1c**, 76-41-5; **4a**, 92078-77-8; **4b**, 92078-78-9; **4c**, 92078-79-0; **5a**, 92078-80-3; **5b**, 92096-20-3; **5c**, 75659-97-1; **6a**, 92078-81-4; **6b**, 92078-82-5; **6c**, 92078-83-6; NH_2OH·HCl, 5470-11-1; $MeONH_2$·HCl, 593-56-6; hydroxylamine-*O*-sulfonic acid, 2950-43-8.

Synthesis and Binding to Tubulin of Colchicine Spin Probes

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Spin probes of deacetylcholchicine (1), 4-(hydroxymethyl)colchicine (2), and colchifoline (3) have been synthesized to study the binding site for colchicine on tubulin. Acylation of 1–3 with (±)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxylic acid (4) afforded diastereomeric mixtures of the esters **5–8** and the amides **9** and **10**. Pure diastereomers of **3** were synthesized with **4a** and **4b**, which inhibited the binding of colchicine by 60%. In the presence of calf brain microtubular protein, the colchifoline spin labels underwent reduction of the nitroxide group, which precluded their use to study the topography of the colchicine binding site.

The antimitotic activity of colchicine is thought to arise from its interaction with tubulin, a protein that polymerizes to form the microtubules of the mitotic spindle. Previous studies have shown that tubulin is a dimer containing nonidentical subunits, one of which has a single colchicine binding site.[1,2] There is currently great interest

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in further characterizing the colchicine binding site on tubulin, since it is thought that this site may also be involved in the antitumor[3a,b] and antigout[4] activity of colchicine. In this regard it may be pointed out that while podophyllotoxin also binds to the colchicine binding site on tubulin, vinblastine interacts with some other site on the protein.[4,5]

Deinum and co-workers[6] have recently employed a spin-labeled analogue of allocolchicine to probe the colchicine binding site of beef brain tubulin. Their results indicated that upon binding to tubulin the spin label became highly immobilized and they suggested that the colchicine binding site was formed by a cleft in the protein. Unfortunately the allocolchicine spin label had a much lower affinity for tubulin than colchicine itself. It was also much less active as an antimitotic agent.

We now report the results of attempts aimed at the preparation of other spin probes, which would be more efficaceous and could possibly constitute better tools to study the microtubule system. These spin probes for the colchicine binding site on tubulin were synthesized from colchifoline (**3**),[7] 4-(hydroxymethyl)colchicine (**2**),[8] and deacetylcolchicine (**1**).[9] Whereas the reaction of **2** and **3** with (±)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxylic acid (**4**) with dehydrating agents affords esters, the reaction of **1** with **4** affords amides, which might bind to tubulin more avidly and selectively. It shall be noted that commercially available **4** gives with **1–3** the optically active derivatives as mixtures of two diastereomers. It was planned to separate such mixtures, if possible, particularly if interesting tubulin binding potency would be found. N-acylation of deacetylcolchicine (**1**) has usually been achieved with acyl chlorides in pyridine or in the presence of potassium carbonate,[10] and esters of colchifoline have been prepared by treating the alcohol **3** with anhydrides in the presence of pyridine.[11] We found that the amidation of **1** and the esterification of **2** and **3** with racemic and optically active **4a** and **4b**[12] with *N,N*-dicyclohexylcarbodiimide (DCC) proceeded more cleanly and in better yield in dry methylene chloride[13] in the presence of 4-(dimethylamino)pyridine as a catalyst.

(±)-2,2,5,5-Tetramethyl-1-pyrrolidinyloxy-3-carboxylic acid (**4**) is a readily available and resolvable chiral nitroxide,[12] useful as a probe in studies of enzyme catalysis[14] and for analyzing biological fluids for drugs.[15] Spin-label studies of enantiomeric specificity in biological systems have been hampered by the lack of optically pure chiral nitroxides of known absolute configuration.

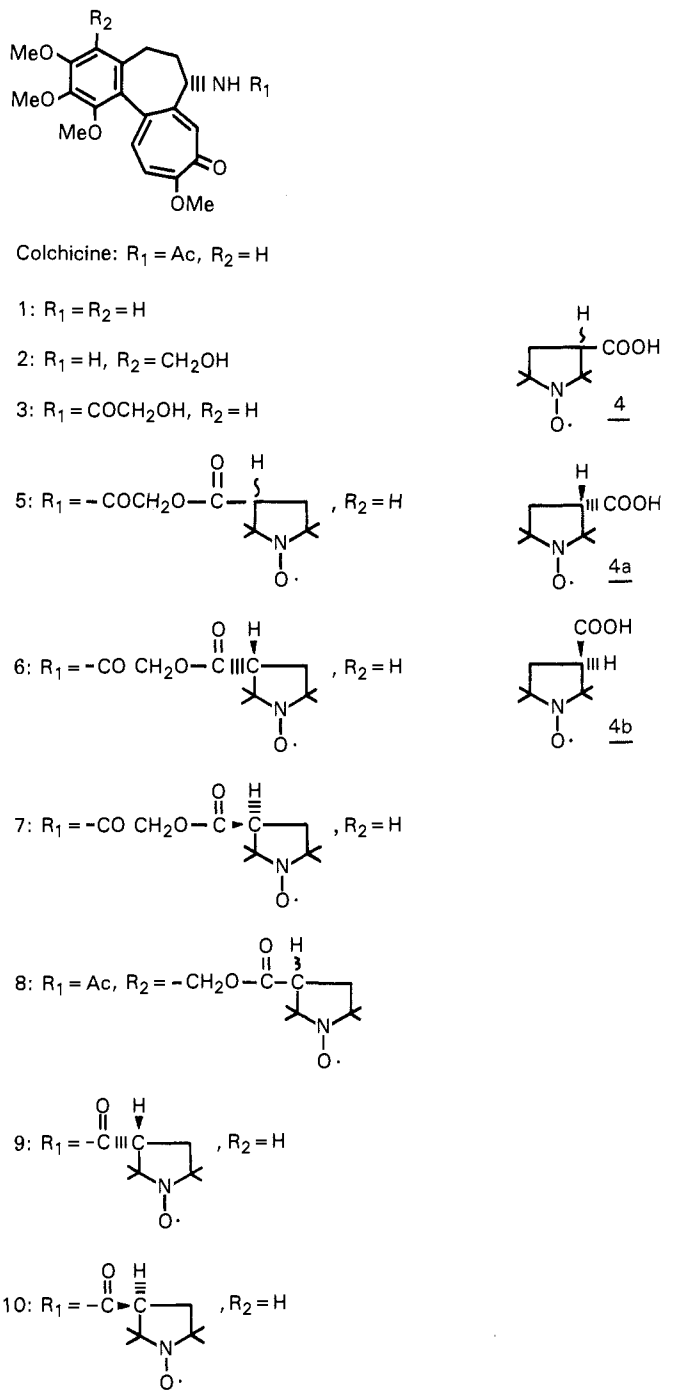

Figure 1.

Treatment of colchifoline (**3**) with equimolar quantities of **4** in dry methylene chloride with a catalytic amount of 4-(dimethylamino)pyridine gave a mixture of diastereomers **5** showing an optical rotation of −107° in chloroform. This mixture could not be separated by TLC (solvent system A–E). The pure diastereomers **6** and **7** (Figure 1) were prepared through a similar reaction with optically

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Table I. Binding of Various Colchicine Spin-Labeled Derivatives to Rat Brain Tubulin Protein

serial no.	compd	% inhib of [^{3}H]colchicine binding[a]
1 ester	**5**	60
2 ester	**6**	72
3 ester	**7**	65
4 ester	**8**	24
5 amide	**9**	0
6 amide	**10**	0
7 amide	colchicine	93

[a] Percentage by which the binding of [^{3}H]colchicine (2.5 μM) to tubulin is reduced in the presence of spin-labeled colchicine analogue (25 μM). Each value is the average of triplicate determinations. For details, see ref 4.

pure acids **4a** and **4b**, respectively, to afford optically pure diastereomers **6** and **7**, exhibiting optical rotations of −106° and −148°, respectively, in chloroform.

The absolute configuration of (*R*)-(+)-**4** (**4a**) has been determined by direct measurement of Bijvoet pairs of reflections[16] and confirmed by a single-crystal study of the (+)-α-methylbenzylamine salt of (*S*)-(−)-**4** (**4b**).[17] Both groups have shown independently that **4a** has the *R* and **4b** the *S* configuration.

The absolute configuration of the pure diastereomers **6** and **7** shown in Figure 1 was assigned in accordance with their optical rotations. An additive effect of optical rotation values in molecules where the centers of chirality are separated by a methylene group has been recognized.[18] Similarly, 4-(hydroxymethyl)colchicine (**2**) on reaction with **4** afforded a diastereomeric mixture of compounds having a different absolute configuration at C_3 of the nitroxide part. This mixture could not be separated by our TLC system, whereas the mixture of the amides obtained from the reaction of deacetylcolchicine (**1**) with **4** could be separated by TLC (silica gel) with $CHCl_3$/MeOH/NH_4OH (95:4:1). The faster running material **9** showed a less negative rotation of −147° and is supposed to be the diastereomer composed of **1** and **4a**, whereas the slower moving compound **10**, with $[\alpha]_D$ −173°, resulted from a combination of **1** with **4b** (Figure 1).

Results of Binding Studies. The ability of the colchicine spin labels to bind to microtubule protein was determined by measuring their ability to displace [^{3}H]-colchicine (Table I) according to the method of Zweig and Chignell.[4] The results clearly show that only the colchifoline spin probes **5**–**7** were active. For the proposed studies, compounds that exhibited tubulin binding less than 50% were arbitrarily not considered to be potent enough. There was no great difference in the binding of the two diastereomers **6** and **7** in comparison to the racemic mixture **5** (Table I).

The two diastereomers **6** and **7** were chosen for the electron spin resonance studies. Since these experiments required the use of large amounts of tubulin, the protein was isolated and purified from calf brain. The ESR spectrum of colchicine spin label **6** in dilute aqueous solution (0.1 M MES buffer, pH 6.5) at room temperature consisted of three sharp lines with a hyperfine splitting of 15.8 G between adjacent peaks (Figure 2). This three-line spectrum results from the hyperfine interaction between the unpaired electron and the nuclear spin of the nitroxide nitrogen atom.[22] When spin-labeled ligands bind to proteins and other macromolecules, their molecular motion decreases.[22] The effect of a decrease in the molecular motion of spin label **6** on its ESR spectrum was investigated by dissolving the label in glycerol and recording the spectrum as a function of temperature. The

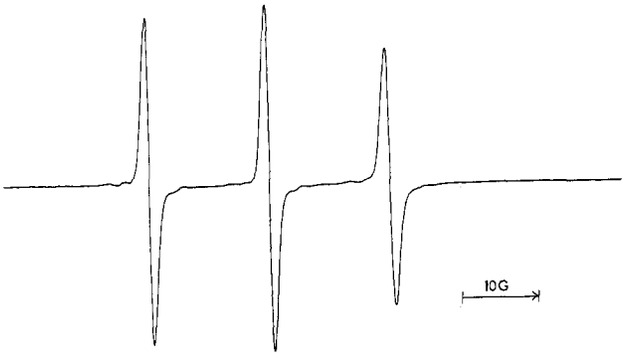

Figure 2. ESR spectrum of colchicine spin label **6** (100 μM) in dilute aqueous solution (0.1 MES buffer, pH 6.5) at room temperature.

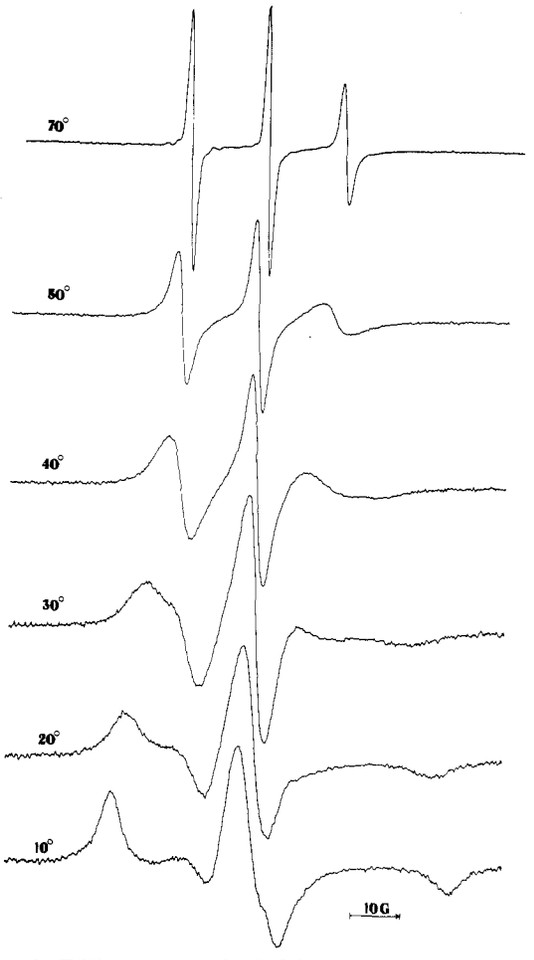

Figure 3. ESR spectrum of spin label **6** (100 μM) M) in glycerol at different temperatures.

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ESR spectrum of label **6** is fairly sharp at 70 °C although the high-field line does exhibit some broadening (Figure 3). On decreasing the temperature of the glycerol solution of **6**, its ESR spectrum begins to broaden further, becoming highly asymmetric below 30 °C (Figure 3).

No immediate change was observed in the ESR spectrum of the spin label **6** on incubating with calf brain tubulin. However, the intensity of the ESR spectrum of **6** decreased with time, the rate being faster at 37 °C than 25 °C. Since there was no concomitant appearance of an ESR spectrum attributable to a bound spin label, it seems reasonable to assume that the observed decrease in the intensity of label **6** was due to the destruction of the nitroxide group. Since the addition of ferricyanide restored the ESR spectrum, it was assumed that reduction of the spin label had occurred. Tubulin is known to contain a number of sulfhydryl groups,[19-21] which are potentially capable of reducing the nitroxide moiety.[23] This reaction, which is catalyzed by heavy metals, particularly iron,[24] may be inhibited by the presence of suitable chelating agents. While the buffer used in the present investigation contained 1 mM EGTA, this chelating agent has a higher affinity for magnesium than iron. The presence of a relatively high concentration of magnesium ions (1 mM) in all incubations may have precluded the chelation of trace amounts of iron and other heavy metals by the EGTA.

Deinum and co-workers[6] were able to observe the ESR spectrum of their allocolchicine spin label bound to microtubular protein by incubating the label with the protein at 37 °C, followed by passage over a Sephadex G-25 column at 4 °C to remove the unbound ligand. The ESR spectrum of the bound allocolchicine spin label, which resembled that recorded for spin label **6** in glycerol at 20 °C (Figure 3), was characteristic of a highly immobilized ligand. When the approach used by Deinum et al. was attempted only a weak signal due to unbound spin label was observed with label **6**. This finding suggests that if label **6** did indeed bind to microtubular protein, then the dissociation rate of the resultant complex was too rapid to permit the separation of the protein-bound label from the free label on the column.

Experimental Section

Melting points were taken on a Fisher-Johns apparatus and are uncorrected. Elemental analyses were performed by the Section on Microanalytical Services and Instrumentation of this Laboratory. Optical rotations were measured in $CHCl_3$ by using a Perkin-elmer Model 141 polarimeter unless otherwise stated. Chemical-ionization mass spectra (CI-MS) were determined by using a Finnigan 1015D spectrometer and a Hitachi Perkin-Elmer RMU-6E spectrometer. Electron-ionization mass spectra (EI-MS) were obtained with a Hitachi Perkin-Elmer RMu-6E spectrometer (70 eV). Thin-layer chromatography plates were purchased from Analtech, Inc., Newark, DE. Solvent systems used for TLC (silica gel) were as follows: (A) CH_2Cl_2/MeOH = 9.5:0.5, (B) $CHCl_3$/MeOH/NH_4OH = 9.5:0.4:0.1, (C) CH_2Cl_2/MeOH = 9.7:0.3, (D) CH_2Cl_2/MeOH 9.5:0.5, (E) $CHCl_3$/MeOH/NH_4OH = 9.5:0.4:0.1.

(±)-2,2,5,5-Tetramethyl-1-pyrrolidinyloxy-3-carboxylic acid (**4**) was purchased from Eastman Kodak Co. Rochester, NY. (+)- and (−)-α-methylbenzylamine were purchased from Aldrich Chemical Co., Milwaukee, WI.

Resolution of (+)-2,2,5,5-Tetramethyl-1-pyrrolidinyloxy-3-carboxylic Acid (4). The racemic acid **4** was resolved following the literature procedure.[12] The salts obtained with (+)- and (−)-α-methylbenzylamine, respectively, were crystallized five times from acetone, to afford after acidification and workup *R*-(+)- and *S*-(−)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxylic acid, respectively (**4a** and **4b**), showing the following properties after drying at 60 °C in high vacuum. (*R*)-(+)-**4** = **4a**: mp 197 °C; $[\alpha]^{25}_D$ +83.5° (*c* 0.4, EtOH). (*S*)-(−)-**4** = **4b**: mp 197 °C; $[\alpha]^{25}_D$ −83.0° (*c* 0.7, EtOH).

Preparation of Esters and Amides of Colchinoids: (−)-*O*-[(2,2,5,5-Tetramethyl-1-oxy-3-pyrrolidinyl)carbonyl]colchifoline (5). A solution of 2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxylic acid (**4**; 55 mg, 0.29 mmol), *N,N*-dicyclohexylcarbodiimide (DCC; 100 mg, 0.46 mmol), colchifoline (**3**; 126 mg, 0.30 mmol), and 4-(dimethylamino)pyridine (10 mg) in dry CH_2Cl_2 (5 mL) was stirred at room temperature for 30 min, until the TLC (silica gel, solvent system A) showed the absence of starting material in the reaction mixture. The reaction mixture was filtered from insoluble materials and the organic layer washed with water (2 × 3 mL), 10% aqueous $NaHCO_3$ (3 × 3 mL), 0.1 N HCl (3 × 3 mL), water (2 × 3 mL), dried (Na_2SO_4), and concentrated to leave a residue which was purified by column chromatography over silica gel and eluted with solvent system C to afford a pure solid, which was triturated with ether to afford a light yellowish solid **5** (65 mg, 37%): mp 125 °C; $[\alpha]^{22}_D$ −107° (*c* 0.5); EI-MS, *m/e* 583 (M^+); CI-MS, *m/e* 584 ($M^+ + 1$). Anal. ($C_{31}H_{39}N_2O_9 \cdot H_2O$) C, H, N.

(−)-*O*-[(2,2,5,5-Tetramethyl-1-oxy-3-pyrrolidinyl)carbonyl]colchifoline (6) from 4a. The method adopted was exactly as described above, by reacting **4a** with **3**, and the ester derivative was purified by column chromatography (silica gel) and eluted with solvent system D to afford **6** (31%): mp 142 °C; $[\alpha]^{25}_D$ −106° (*c* 0.2); EI-MS, *m/e* 583 (M^+). Anal. ($C_{31}H_{39}N_2O_9$) C, H, N.

(−)-*O*-[(2,2,5,5-Tetramethyl-1-oxy-3-pyrrolidinyl)carbonyl]colchifoline (7) from 4b. This ester was prepared similarly to **5** by reacting **4b** with **3**; purification of the ester derivative afforded **7** (36%): mp 136 °C; $[\alpha]^{25}_D$ −148° (*c* 0.2); EI-MS, *m/e* 583 (M^+). Anal. ($C_{31}H_{39}N_2O_9 \cdot 1^1/_2H_2O$) C, H, N.

(−)-*O*-[(2,2,5,5-Tetramethyl-1-oxy-3-pyrrolidinyl)carbonyl]-4-(hydroxymethyl)colchicine (8). The method used to prepared **8** was essentially the same as described for **5**, except that catalytic amounts of 4-pyrrolidinopyridine were used instead of 4-(dimethylamino)pyridine. The residue obtained after workup was crystallized from a mixture of petroleum ether–ether to afford **8** as a solid (34%): mp 141 °C; $[\alpha]^{21}_D$ −43.5° (*c* 0.8); CI-MS, *m/e* 598 ($M^+ + 1$). Anal. ($C_{32}H_{41}N_2O_9 \cdot 1^1/_2H_2O$) C, H, N.

(−)-*N*-[(2,2,5,5-Tetramethyl-1-oxy-3-pyrrolidinyl)carbonyl]deacetylcolchicine (9) and Its Diastereomer 10. The method used was the same as described for the preparation of **5**. The crude residue obtained after the usual workup was purified by column chromatography over silica gel and the amide was eluted with solvent system E to afford a faster moving material, which was collected and crystallized with a mixture of CH_2Cl_2–ether to afford a solid **9** (28%): mp 170 °C; $[\alpha]^{25}_D$ −147° (*c* 0.5); CI-MS, *m/e* 526 ($M^+ + 1$). Anal. ($C_{29}H_{37}N_2O_7 \cdot H_2O$) C, H, N.

The slower moving material was crystallized with a mixture of CH_2Cl_2–ether to afford **10** (26%): mp 297 °C; $[\alpha]^{25}_D$ −173° (*c* 0.5); CI-MS, *m/e* 526 (M^+ 1). Anal. ($C_{29}H_{37}N_2O_7 \cdot 2H_2O$) C, H, N.

Isolation of Microtubule Protein. Microtubular protein was purified from fresh calf brain by using successive cycles of polymerization and depolymerization.[25] The microtubular protein was stored at −20 °C in 0.1 M MES buffer, pH 6.5 (0.1 M 2-*N*-morpholinylethanesulfonic acid, 1 mM ethylene glcyol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 0.5 mM $MgCl_2$) containing 2 M glycerol. Protein was determined by the biuret method,[26] with bovine serum albumin as a standard. The purity of the microtubular protein was determined by using polyacrylamide gel electrophoresis.[27]

Electron Spin Resonance Studies. ESR measurements were made on a Varian E-104 spectrometer operating at 9.5 GHz. In

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a typical experiment the spin-labeled colchicine analogue ($1–5 \times 10^{-5}$ M) in 0.1 M MES buffer containing 0.5 mM GTP was incubated at either 25 or 37 °C, for 1.5 h with an equal concentration of purified calf brain tubulin. The sample was then transferred to a quartz aqueous flat cell and the ESR spectrum recorded using the following instrumental parameters: microwave power, 20 mW; modulation, 2 g; scan time, 4 min; scan range, 100 G. In some experiments the sample was transferred immediately to the flat cell and the spectrum recorded as a function of time at either 25 or 37 °C. In other experiments the spin label (5×10^{-5} M) and tubulin (5×10^{-5} M) were incubated at 37 °C for 1.5 h and then passed over a short column (1×25 cm) of Sephadex G-25 and the column eluted at 4 °C with 0.1 M MES buffer. The fractions were then placed in the flat cell and their ESR spectra recorded.

Microtubule Binding Assay. The binding of the spin labels to rat brain microtubular protein was measured as previously described.[4] Briefly, the method consisted of incubating a 200-μL aliquot of rat brain supernatant containing about 1.0 mg of protein, with 800 μL of a solution containing 10 mM sodium phosphate buffer (pH 7.0), 5 mM $MgCl_2$, 0.1 mM GTP, 240 mM sucrose, and 2.5 μM [^{3}H]colchicine (0.2 Ci/mmol; New England Nuclear). The colchicine analogues were added at the beginning of the incubation to a final concentration of 25 μM. After a 2-h incubation at 37 °C, the reaction was stopped by the addition of 1 mL of an ice-cooled solution containing 100 μM colchicine. The [^{3}H]colchicine complex was isolated by adsorption onto DE 81 Whatman Chromedia filter paper. After washing the filters were transferred to counting vials, 10 mL of Aquasol (New England Nuclear) was added, and the sample was counted in a Mark III Searle scintillation counter. The results for the colchicine analogues were expressed in terms of the percent inhibition of [^{3}H]cholchicine binding. Under our assay conditions, the addition of 25 μM unlabeled colchicine to the incubation decreased [^{3}H]colchicine binding by about 90%.

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Bis(*m*-nitrophenyl) and Bis(*p*-nitrophenyl) Esters and the Phosphorodiamidate of Thymidine 5′-Phosphate as Potential Sources of Intracellular Thymidine 5′-Phosphate in Mouse Cells in Culture

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Thymidine 5′-phosphate (TMP) derivatives with masked phosphate groups were synthesized in tritiated form from [*methyl*-^{3}H]thymidine. They were of interest as models for 5′ nucleotide derivatives that might be able to permeate mammalian cells and then liberate intracellular antimetabolite 5′ nucleotides by loss of the masking groups. Mouse L fibroblasts were grown in vitro in the presence of 1 mM 5′-amino-5′-deoxythymidine, which was found to suppress >99% of cellular thymidine kinase activity while inhibiting the rate of cell division by only 30%. The TMP derivatives were less effective than thymidine in labeling the deoxyribonucleic acid (DNA) of the L cells. The labeling was inhibited 95–99% by 5′-amino-5′-deoxythymidine, indicating that it represented incorporation into DNA of [^{3}H]thymidine formed from degradation of the test compounds. No evidence was obtained that the compounds acted as sources of intracellular TMP by cell permeation followed by loss of phosphate blocking groups. Similar studies yielded no evidence that the bis(*m*-nitrophenyl) ester of TMP produced intracellular TMP by that route in the LM(TK$^-$) strain of L cells that are genetically deficient in thymidine kinase.

The development of prodrugs that can act as extracellular sources of intracellular 5′ mononucleotides is of chemotherapeutic interest due to extensive evidence that the growth-inhibitory action of almost all antineoplastic purine and pyrimidine base and nucleoside analogues requires their anabolism, intracellularly, to the corresponding 5′ mononucleotides and that the intracellular level of these nucleotides in drug-resistant neoplasms if frequently low.[1] The nucleotides are poorly membrane permeable,[2–4] are dephosphorylated by plasma enzymes,[5–7] and have been chemotherapeutically ineffective against resistant neoplasms. The ability of various types of potential nucleotide prodrugs to inhibit resistant neoplasms has been studied, but as yet only partial successes have been reported.[8] In the present study we synthesized thymidine 5′-phosphate (TMP, **3**) derivatives that possessed masked phosphate groups and were radioactively labeled in the thymidine (TdR) moiety and utilized them as models of candidate prodrugs of antimetabolite 5′ nucleotides. They were evaluated by bioassay of incorporation of the radioactive TdR into deoxyribonucleic acid (DNA) in cultured cells in which TdR phosphorylation was blocked genetically or by an enzyme inhibitor. Under these conditions, labeling of DNA would be expected only as a result of the liberation of intracellular TMP by loss of the masking groups. TMP diesters (**1a,b**) with lipophilic and electron-withdrawing esterifying groups were studied in view of their potential to hydrolyze, intracellularly, to monoesters (**2a,b**) that might furnish intracellular **3** by phosphodiesterase action. The diamidate **1c** of TMP was also studied because of recent evidence that the diamidate of 5-fluoro-2′-deoxyuridine 5′-phosphate (FdUMP) may be capable of gener-

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