Biological Effects of Modified Colchicines. Improved Preparation of 2-Demethylcolchicine, 3-Demethylcolchicine, and (+)-Colchicine and Reassignment of the Position of the Double Bond in Dehydro-7-deacetamidocolchicines

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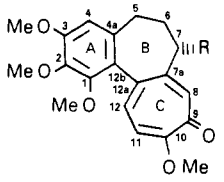
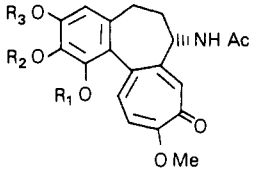
A variety of colchicine, demecolcine, and isocolchicine derivatives were examined for their potency in the lymphocytic leukemia P388 screen in mice, for their toxicity in mice, and for their binding to microtubule protein. A qualitatively direct correlation was found between in vivo potency and toxicity; potency appeared to be less well correlated with tubulin binding. The most potent compounds were N-acylated analogues of colchicine and demecolcine. Among the monophenols, only 3-demethylcolchicine showed an appreciable effect in vitro and in vivo and was less toxic than colchicine. Improved methods were found for the preparation of 3- and 2-demethylcolchicine, which involved the use of 85% phosphoric acid and concentrated sulfuric acid, respectively. Decoupling experiments with ^1H NMR proved that the double bond of dehydro-7-deacetamidocolchiceine and its derived tropolonic methyl ethers **24** and **25** was in the 5,6 position, rather than the 6,7 position formerly tentatively assigned.

The observed antimitotic activity of colchicine (**1**) is





1, R = $NHCOCH_3$

2, R = $NHCH_3$

3, R = $N(CH_3)COCH_3$

4, R = $NHCOCF_3$

5, R = $N(CH_3)CHO$

6, R = $N(CH_3)COCF_3$

7, R = $N(CH_3)CH_2(2\text{-}(OH)C_6H_4)$

8, R = $N(CH_3)_2$

9, R = NH_2

10, R_1 = H; $R_2 = R_3 = CH_3$

11, $R_1 = COCH_3$; $R_2 = R_3 = CH_3$

12, $R_1 = R_3 = CH_3$; R_2 = H

13, $R_1 = R_2 = CH_3$; R_3 = H

14, $R_1 = R_2 = CH_3$; R_3 = Glucosyl

probably the result of its interaction with tubulin, a protein which aggregates to form the microtubules of the mitotic spindle.[2,3] This interaction disrupts a dynamic equilibrium between tubulin and its polymeric form, the microtubule,[4-7] and that interaction has also been said to be responsible for the antigout activity of the drug.[8]

Colchicine (**1**) and demecolcine (**2**) (alkaloids from *Colchicum autumnale*) have been used clinically in certain forms of leukemia and against solid tumors, but their effects were only observed at toxic or near toxic levels.

The object of this report is to try to discern which of the structural entities are needed for optimal bioligical effects and to find the effect of a molecular change on the toxicity, tubulin binding, and antileukemic activity of the resultant compound, as compared with colchicine.

The colchicine molecule, which was first synthesized in 1961,[9] has at least three chemically different sites at which molecular changes are possible, without destruction of the tricyclic system. These are, the three neighboring methoxy groups in the aromatic ring A, the side chain at the chiral center C_7 of ring B, and the tropolone ether moiety in ring C. Structural variations of colchicine demonstrated that the natural arrangement of the tropolone ether function is critical.[3,10] Colchiceine and isocolchicine derivatives were found to be less biologically active, but the methoxy group in the C ring of colchicine could be replaced by a variety of nucleophiles (e.g., amines and thiols) without loss of activity.[11]

It seemed logical to start with appropriate changes in ring B, followed by variations in rings A and C. Colchicine and isocolchicine derivatives with different acylamido side chains at C_7 (**3–6**, **15**, **17**, and **18**) were examined, as well

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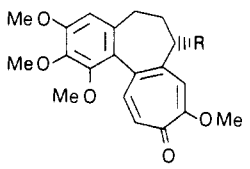
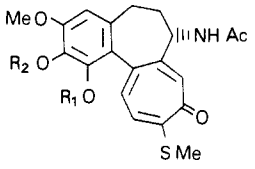




15, R = $NHCOCF_3$

16, R = $N(CH_3)_2$

17, R = $N(CH_3)CHO$

18, R = $N(CH_3)COCH_3$

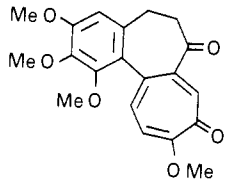
19, $R_1 = R_2 = CH_3$

20, $R_1 = CH_3CO$; $R_2 = CH_3$

21, $R_1 = H$; $R_2 = CH_3$

22, $R_1 = CH_3$; $R_2 = CH_3CO$

as amines (2, 7–9, and 16) and compounds without nitrogen at C_7 (23–25). (+)-Colchicine (26) was resynthesized to



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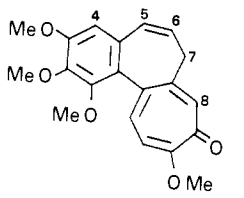

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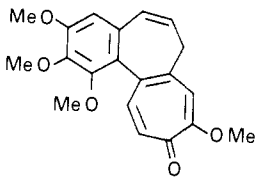

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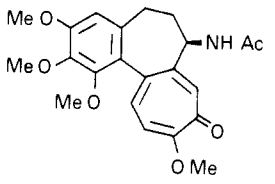
determine the influence of chirality on toxicity and binding affinity to tubulin. The biological effects of demethylations in ring A were examined with the three isomeric phenols (10, 12, and 13) and the acetate (11). Finally, thiocolchicine and derivatives (19–22), the glucoside alkaloid colchicoside (14), demecolceine (27), and precolchicine (28), a colchicine-like molecule with a rearranged double-bond system, were included in the studies.



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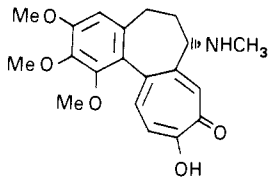

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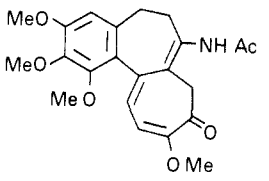

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Chemistry. The syntheses of most of the compounds in Table I were previously described[12-16] (for details see Experimental Section).

N-Formyldemecolcine (5)[17,18] and *N*-formylisodemecolcine (17) were prepared by refluxing demecolcine (2) and isodemecolcine[12] in ethyl formate. Improved procedures were developed for the syntheses of 2-demethylcolchicine (12), 3-demethylcolchicine (13), and (+)-colchicine (26).

2-Demethylcolchicine (12), first prepared from colchicine (1) by Bladé-Font[15] with $AlCl_3$ in CH_2Cl_2 via the acetate in about 20% yield, was synthesized in a convenient one-step reaction. Colchicine and derivatives are extremely sensitive to dilute acids, resulting in the cleavage of the tropolone ether. More concentrated acids give, additionally, hydrolysis of the amide side chain. However, by examining numerous acids, we found that the selective cleavage of the 2-methoxy group of colchicine could, surprisingly, be achieved in concentrated sulfuric acid at 60 °C. Very little cleavage of the tropolonic ether was observed, and 2-demethylcolchicine (12) could be isolated in 40–50% yield.

3-Demethylcolchicine (13) had been previously directly prepared only by enzymatic hydrolysis of colchicine (1)[19,20] or colchicoside (14).[21] We sought a mild and selective chemical method to cleave the glucoside bond in 14 and found that trifluoroacetic acid gave a small amount of the wanted phenolic compound (13). This prompted us to try other acids as well. Thus, treatment of colchicoside (14) with 85% phosporic acid at room temperature afforded 3-demethylcolchicine (13) in high yield.

The preparation of (+)-colchicine (26) was greatly facilitated by Bladé-Font's new racemization procedure.[22] Acid hydrolysis[23] of (±)-colchicine and optical resolution, N-reacetylation, O-methylation, and separation from (+)-isocolchicine followed in principle published procedures[24] and ultimately afforded optically pure (+)-colchicine (26).

The position of the double bond in the tropolone dehydro-7-deacetamidocolchiceine[9] and in the derived two isomeric tropolonic methyl ethers 24 and 25[13] had not been previously determined. Our reinvestigation of this problem revealed that the tentative assignment of a 6,7 double bond had to be corrected.[9,13]

Repetition of ^1H and ^{13}C NMR studies confirmed our former data.[13] However, decoupling of the methylene protons by irradiation yielded a 40% peak-height enhancement of the tropolonic C_8 H singlet, while the intensity of the C_4 H proton on the benzene ring was unaffected. This clearly demonstrates that the methylene protons are close to the ring C proton at C_8, not to the ring A proton at C_4. Since the same result was found for both isomeric tropolonic methyl ethers 24 and 25, as well as for the parent tropolone, the double bond in all three compounds must be located in the 5,6 position.

Structure–Activity Relationships. It can be seen in Table I that a good correlation exists between the in vivo potency of these compounds in the lymphocytic leukemia P388 screen of NCI (see Experimental Section) and their

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Table I. Potency in P388 Lymphocytic Leukemia Screen, Tubulin Binding, and Toxicity of Colchicine and Derivatives

no.	compound	potency,[a] μmol/kg	tubulin binding,[b] %	toxicity (NCI)[c]	toxicity (LC)[d]
1	colchicine	0.4[e]	90	2.5	3.0 (2.5-4.0)
3	*N*-acetyldemecolcine	0.4[e]	98	3.0	3.6 (2.4-5.1)
4	*N*-deacetyl-*N*-(trifluoroacetyl)colchicine	0.4[e]	100	0.9	1.1 (0.9-1.3)
6	*N*-(trifluoroacetyl)demecolcine	1.5[e]	83	5.4	8.8 (6.4-11.8)
5	*N*-formyldemecolcine	1.7[e]	76	6.3	179.7 (101.5-317.8)
13	3-demethylcolchicine	3.6	68	26.0	31.7 (22.6-44.4)
23	7-deacetamido-7-oxocolchicine	9.6[e]	59	28.1	
2	demecolcine	10.8	93	21.6	236.7 (200.8-279.0)
12	2-demethylcolchicine	13.8[e]	50	51.9	42.9 (28.6-63.9)
24	5,6-dehydro-7-deacetamidocolchicine	15.9	90	29.4	~300-600[f]
7	speciosine	22.0	62	83.9	296.4 (227.9-385.7)
8	*N*-methyldemecolcine	68[g]	38	51.9	519.5 (337.7-797.4)
10	1-demethylcolchicine	86[h]	26	103.9	>520[f]
19	thiocolchicine		90		
22	2-demethyl-2-acetylthiocolchicine		78		
20	1-demethyl-1-acetylthiocolchicine		67		
11	1-demethyl-1-acetylcolchicine	inact	59	46.8	205.9 (166.3-254.8)
9	*N*-deacetylcolchicine	inact[e]	51	28.0	137.0 (106.2-176.8)
15	*N*-deacetyl-*N*-(trifluoroacetyl)isocolchicine	inact	18		
16	*N*-methylisodemecolcine	inact	13		
17	*N*-formylisodemecolcine	inact	5		
25	5,6-dehydro-7-deacetamidoisocolchicine	inact	0		
28	precolchicine	inact	0		
21	1-demethylthiocolchicine		0		
14	colchicoside	inact			
27	demecolceine	inact	0		
18	*N*-acetylisodemecolcine	inact	0		
26	(+)-colchicine		32		122.6 (82.9-163.4)

[a] Obtained by graphical estimation of the potency which would give a T/C = 140. [b] Percentage by which binding is reduced by the presence of the inhibitor, at 25 μM with [^{3}H]colchicine at 2.5 μM. Values are the average of triplicate assays. [c] Toxicity in the P388 NCI screen. Defined as that dose, in μmol/kg, which caused an average weight loss > 3 g in a set of mice or the death of one or more of the mice in the set. Deaths were noted on day 5 of a series of nine injections in 9 days (1 per day) of the compound. [d] Toxicity found after a single im injection, in μmol/kg. The total number of deaths were counted after 7 days at various dose levels, and the LD_{50} was determined by probit analysis. Parenthesized numbers represent 95% confidence interval. [e] Confirmed by a second P388 assay. [f] Insufficient data for probit analysis. [g] Extrapoiated from T/C = 130 at 20, 124 at 10, and 117 at 5 mg/kg. [h] Obtained from a single determination (T/C = 167 at 56 mg/kg).

toxicity in that screen. With few exceptions, molecular modification which produced compounds with potencies equivalent to or less than colchicine also resulted in correspondingly altered toxicities.

Variations in structure which resulted in compounds having potencies similar to that of colchicine can be seen, in Table I, to be embodied in the secondary and tertiary amides **4**, **3**, **5**, and **6**. These compounds have *N*-acetyl, *N*-(trifluoroacetyl) and *N*-formyl groups in the colchicine or demecolcine series. The *N*-(trifluoroacetyl) compound **4** was an exception to the direct relationship of potency to toxicity among these colchicine derivatives; it was more toxic than expected.

Very toxic compounds showed qualitatively good correlation between the toxicities determined through a single im injection [toxicity (LC) in Table I] and those found during the multiple dose NCI studies [toxicity (NCI) in Table I] (see Experimental Section). Less toxic compounds had a wider spread between the two numbers. It is reasonable to expect the "toxicity (NCI)" values to be lower than "toxicity (LC)" because the multiple doses used in the P388 assay tend to increase the toxic effect of the drug, perhaps due to bioaccumulation. The single injection toxicity (LC) determinations were of value in indicating the relative toxicities of the set of compounds before the longer-term P388 assay was employed.

It can be observed, in Table I, that a direct relationship between tubulin binding (see Experimental Section) and in vivo potency in the P388 system is not generally obtained. Thus, demecolcine (**2**) and 5,6-dehydro-7-deacetamidocolchicine (**24**) bind as well as colchicine in the tubulin assay but are one or two orders of magnitude less potent than colchicine in the P388 assay. These results are not surprising when it is realized that other factors besides microtubule binding, e.g., absorption, distribution, metabolism, and excretion, are also important in the in vivo studies. However, the tubulin assay may be very useful if looked at as follows. Compounds which show less than ca. 50% tubulin binding always appear to be inactive, or almost so, in the P388 screen. Those compounds which show affinity between the 50 and 60% levels may have some in vivo activity, and compounds with affinity greater than 60% are worthy candidates for the P388 assay.

3-Demethylcolchicine (**13**) alone among the aromatic ring A demethylated compounds retains considerable potency in the P388 assay. Its potency was within an order of magnitude of the potency of colchicine. The 1-methoxy and 2-methoxy groups are, apparently, essential for both in vivo potency and tubulin binding. It is possible that there is a hydrogen-bonding moiety on the receptor, at some distance from the 1- and 2-methoxy groups. Conversion of these methoxy groups to the phenolic compound could cause the molecule to change its orientation sufficiently to allow H bonding between that phenolic group and some moiety in tubulin and remove the ring C oxygen atoms from their critical binding area, lowering overall affinity in tubulin binding. A direct relationship between tubulin binding and P388 potency can be observed in the 3-demethyl (**13**), 2-demethyl (**12**), and 1-demethyl (**10**) series. Their in vitro and in vivo potencies are also directly related, qualitatively, to in vivo toxicities; as the potency and tubulin binding affinity increase, so does the toxicity.

Conversion of the ring C methoxy group to a thiol ether does not lessen tubulin binding. Also, thiocolchicine (**19**)

is more effective in vivo in the P388 screen than colchicine itself.[25] Its toxicity is known to be greater than colchicine in this assay, however. The interchange of sulfur for oxygen in the third site of the colchicine molecule would not appear to be as advantageous as changes at C_3 in ring A or C_7 in ring B if a major concern was decreased toxicity.

All other changes in the colchicine molecule appeared to be disadvantageous. Conversion of the amide moiety to a secondary or tertiary amine caused a considerable loss of biological potency or its total loss. Conversion to any isotropolonic ether structure in ring C completely abolished activity. A change in the double-bond pattern, as in precolchicine, eliminated its affinity for tubulin binding.

The chirality of colchicine is, evidently, recognized by several biological substrates. (+)-Colchicine (**26**) binds less well to tubulin and albumin[26] and is less toxic than its natural enantiomer (Table I).

Certain changes in rings A and B of the colchicine molecule have been shown to lower the toxicity of the parent molecule, albeit these compounds also display lowered potency. It is possible, however, that combinations of changes in the various rings, i.e., *N*-acetyl- or *N*-formyl-3-demethyldemecolcines, will produce compounds with sufficient in vivo potency and markedly lowered toxicity. Synthesis of these types of compounds is in progress and will be the subject of future papers.

Experimental Section

Chemical Methods. Melting points were determined on a Thomas-Hoover capillary apparatus. Elemental analyses were performed by the Section on Microanalytical Services and Instrumentation of this Laboratory. IR spectra were obtained on a Beckman 4230 and mass spectra on a Hitachi RMU-6E (70 eV) and Finnegan 1015D (CI). ^1H NMR spectra were obtained (using tetramethylsilane at 0.0 ppm as internal reference) on a Varian HR-220 spectrometer or a JEOL FX 100 spectrometer. ^1H and ^{13}C NMR spectra of **24** and **25** were run in Fourier-transform mode on a Varian XL-100 spectrometer at a probe ambient temperature of 27 °C. Samples (2 mg each for ^1H and 30–40 mg for ^{13}C) were dissolved in 0.35 mL of $CDCl_3$ containing Me_4Si as internal reference, and homonuclear proton decoupling experiments were performed on the ^{13}C NMR solutions in the continuous-wave mode. UV spectra were measured on a Beckman DB-G grating spectrophotometer. Optical rotations were measured on Perkin-Elmer 141 and 241 MC polarimeters.

Origin of Tested Compounds. Colchicine (**1**) was purchased from Fluka AG (Switzerland) or Aldrich Chemical Co. (WI) and colchicoside (**14**) from Fluka AG. Compounds (**19–22** and **28**) were submitted by Dr. A. Bladé-Font, Productos Frumtost, S.A., Barcelona, Spain. Thiocolchicine (**19**) was obtained from Dr. P. Bellet, Roussel-Uclaf, Paris, France. The synthesis of the following compounds was reported earlier: **2**, **4**, **6–9**, **15**, **18**, **27**;[12] **3**, **16**, **24**, **25**;[13] **10**, **11**;[14,15] and **23**.[16]

***N*-Formylisodemecolcine (17).** A solution of isodemecolcine[12] (740 mg, 2 mmol) in 6 mL of ethyl formate was refluxed for 24 h. After evaporation of solvent, the residue was filtered over SiO_2 with $CHCl_3$/MeOH (95:5), giving an oil (375 mg, 47%) which crystallized on addition of Et_2O. Recrystallization from EtOAc/Et_2O afforded pure **17**: mp 208–211 °C; $[\alpha]^{22}_D$ −253.4° (*c* 1.09, $CHCl_3$). The ^1H NMR spectrum of this compound showed the presence of formyl rotamers: NMR ($CDCl_3$) δ 2.10–2.80 (m, 4, CH_2CH_2), 2.86 and 3.23 (2 s, ca. 2:1 rotamer ratio, 3, NCH_3), 3.69 and 3.71 (2 s, 3, C_1 MeO), 3.92 (s, 9, $C_{2,3,9}$ MeO), 4.18 and 4.66 (2 m, 1, C_7 H), 6.57–6.70 (m due to rotamers, 2, C_4 H and C_8 H), 7.14 and 7.11 (2 d, 1, C_{11} H, J = 12 Hz), 7.38 and 7.41 (2 d, 1, C_{12} H, J = 12 Hz), 8.40 and 8.23 (2 s, 1, CHO); IR ($CHCl_3$) 1665 (CHO), 1621, 1599 and 1569 (tropolonic ether) cm^{-1}; MS, *m/e* 399. 371 (base), 356, 340, 312.

***N*-Formyldemecolcine (5).** This compound was prepared in the same manner as **17**. The physical data of **5** were identical with those previously reported.[17,18,27]

2-Demethylcolchicine (12). A mixture of 500 mg (1.25 mmol) of colchicine (**1**) and 2.5 mL of concentrated H_2SO_4 was stirred at 60 °C for 5 h. The reaction can be monitored by TLC [small drop of reaction mixture diluted with MeOH, applied to SiO_2 plate, CH_2Cl_2/MeOH (90:10)]. The yellow solution was poured on ice, adjusted to pH 5 under cooling with 2 N NaOH, and extracted with CH_2Cl_2. The extracts were dried (Na_2SO_4), evaporated, and chromatographed on Al_2O_3 (basic, Alfa Division, Danvers, MA) by elution of small amounts of less polar products with CH_2Cl_2/MeOH (99:1), followed by **12** with CH_2Cl_2/MeOH (95:5 and 90:10). Evaporation of fractions containing **12** afforded an oil, which was crystallized from $CHCl_3$ to give 290 mg (46%) of 2-demethylcolchicine (**12**) as $CHCl_3$ solvate: mp 172–180 °C (lit.[28,15] 176–182 and 190–200 °C), $[\alpha]^{25}_D$ −135° (*c* 1, $CHCl_3$) (lit.[28,15] $[\alpha]^{22}_D$ −130.7° (*c* 2.0118, $CHCl_3$) and −137° (*c* 1, $CHCl_3$).

Physical data (NMR, IR, UV, and MS) of this compound were identical with those reported earlier for natural 2-demethylcolchicine[28] and synthetic 2-demethylcolchicine.[15]

3-Demethylcolchicine (13). A mixture of 250 mg (0.46 mmol) of colchicoside (**14**) and 2 mL of 85–88% phosphoric acid was stirred at 25 °C for 24 h. The yellow solution was diluted with 10 mL of water, adjusted to pH 5 by addition of 2 N NaOH, and extracted with CH_2Cl_2. The extracts were dried (Na_2SO_4), evaporated, and chromatographed on Al_2O_3 (basic, Alfa Division, Danvers, MA) first with CH_2Cl_2/MeOH (99:1) followed by CH_2Cl_2/MeOH (95:5). The appropriate fractions after evaporation and crystallization from a small amount of acetone afforded 180 mg (89%) of 3-demethylcolchicine (**13**) as acetone solvate: mp 171–177 °C, resolidification 205–210 °C, second mp 270 °C dec (lit.[29] mp 180–190 and 275–280 °C); $[\alpha]^{25}_D$ −151° [*c* 1 (solvate crystals), $CHCl_3$] and −250° [*c* 1 (solvate crystals), MeOH] [lit.[29] $[\alpha]_D$ −128° (*c* 1, $CHCl_3$) and −231° (*c* = 1, MeOH)].

The different optical rotations can be explained with solvent and concentration effects due to different solvates being measured. These effects are well known from the studies of Bellet and Regnier.[30] The spectral data (NMR, IR, UV, and MS) of 3-demethylcolchicine (**13**) prepared by the new method were identical with those of an authentic sample of this compound.[31]

(+)-Colchicine (26). A solution of (−)-colchicine (**1**; 10 g, 25 mmol) in 100 mL of acetic anhydride was refluxed for 48 h and, after addition of 17 mL of H_2O, for an additional 10 h.[22] The solvent was removed in vacuo and the residue was crystallized from ethyl acetate, giving 7.4 g of (±)-colchicine: mp 235–245 °C; $[\alpha]^{20}_D$ 0° (*c* 1, $CHCl_3$). Acid hydrolysis of this compound, following the procedure of Fernholz,[23] afforded in 80% yield (±)-*N*-deacetylcolchiceine, mp 244–246 °C (lit.[24] 244 °C). Optical resolution following the procedure of Corrodi and Hardegger[24] gave (+)-*N*-deacetylcolchiceine. (+)-*N*-Deacetylcolchiceine (0.95 g) in 40 mL of benzene and 2 mL of acetic anhydride was stirred at 50–60 °C for 1 h. Evaporation of solvents gave (+)-colchiceine: mp 156–161 °C (H_2O); $[\alpha]^{20}_D$ +250.5 (*c* 1, $CHCl_3$) [lit.[24] mp 152 °C; $[\alpha]_D$ +251° (*c* 1, $CHCl_3$)]. Methylation of this compound with ethereal diazomethane afforded a mixture of (+)-colchicine and (+)-isocolchicine, which were separated on SiO_2 with $CHCl_3$/MeOH (97.5:2.5). (+)-Colchicine (**26**) was eluted first and recrystallized from H_2O, mp 139–141 °C [lit.[24] mp 140 °C (softening)]. This compound was indistinguishable from the natural compound (NMR, TLC), except for its opposite optical rotation, $[\alpha]^{20}_D$ +131° (*c* 0.87, $CHCl_3$) [lit.[32] for (−)-colchicine (**1**): $[\alpha]^{17}_D$ −121° (*c* 1, $CHCl_3$)].

Reassigned NMR data[13] of 5,6-dehydro-7-deacetamidocolchicine (24): ^1H NMR ($CDCl_3$) δ 6.54 (s, 1, C_4 H), 6.50 (d,

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1, C_5 H, J = 8.5 Hz), 6.18 (m, 1, C_6 H), 3.06 (d, 2, C_7 $H_{ax}H_{eq}$, J = 6.3 Hz), 7.29 (s, 1, C_8 H); ^{13}C NMR ($CDCl_3$) δ 106.6 (d, C_4), 129.4 (d, C_5), 130.2 (d, C_6), 38.9 (t, C_7), 133.5 (d, C_8).

Reassigned NMR data[13] of 5,6-dehydro-7-deacetamidoisocolchicine (25): ^{1}H NMR ($CDCl_3$) δ 6.62 (s, 1, C_4 H), 6.59 (d, 1, C_5 H, J = 9.0 Hz), 6.25 (m, 1, C_6 H), 3.09 (dd, 1, C_7 H_{eq}, $^2J_{gem,7_{ax},7_{eq}}$ = 12.5 Hz, $^3J_{vic,6,7}$ = 7.5 Hz), 2.65 (ddd, 1, C_7H_{ax}, $^2J_{gem,7_{ax},7_{eq}}$ = 12.5 Hz, $^3J_{vic,6,7}$ = 6.0 Hz, $^4J_{allylic,5,7}$ = 1.5 Hz), 6.81 (s, 1, C_8 H); ^{13}C NMR ($CDCl_3$) δ 106.3 (d, C_4), 129.3 (d, C_5), 130.7 (d, C_6), 38.6 (t, C_7), 115.5 (d, C_8).

In the decoupling experiment of the anisochronous methylene protons in **25**, the 40% peak-height enhancement of C_8 H consisted of 30% from the irradiation of the lower-field equatorial methylene proton C_7 H_{eq} and of 10% from the higher-field axial proton C_7 H_{ax}. The peak-height enhancement arose from decoupling of the unresolved $^4J_{allylic,7,8}$ homonuclear proton spin–spin coupling and demonstrated that the methylene protons were close to the tropolonic C_8 H proton and not to the C_4 H proton on the benzene ring.

Biological Methods. P388 Assay. This assay was carried out by the Drug Evaluation Branch of the Division of Cancer Treatment at NCI. The P388 test system employed 20–22 g mice with lymphocytic leukemia. A multiple dose assay was used with one injection (im) per day for 9 days. The compounds were introduced in hydroxypropylcellulose suspension or in aqueous solution. Compounds which showed reasonable activity in the initial assay were reassayed for confirmation of that activity.

In order to permit an easier comparison of the potencies of the compounds in Table I, a potency, in milligrams per kilogram, was calculated[25] for an arbitrary value of T/C = 140 (a 40% increase in survival time in a treated vs. a control set of mice in the NCI P388 assay). Only one of the active compounds did not reach the T/C = 140 at any dose level [*N*-methyldemecolcine (8)]. All of the other active compounds had T/C ≥140 at one, or more, dose levels. The "T" represents the average number of days the group of treated mice survived at a particular dose level in the assay, and "C" is the survival time of a group of control mice. Compounds with T/C < 120, at any nontoxic dose level, were considered to be inactive.

To find the dose level, in milliograms per kilogram, required to give a T/C = 140, and thus ascertain the relative potencies of the various compounds at this arbitrary value, the T/C was plotted against the dose level for each compound. Thus, the dose, in milligrams per kilogram, necessary to obtain a T/C = 140 was obtained from the manually drawn line through the experimentally determined points. Extrapolation was necessary only for 8. The dose was converted from milligrams per kilogram to micromoles per kilogram for Table I. T/C values obtained from NCI are not completely reproducible, quantitatively. The variation in T/C values between assays was such that, qualitatively, compounds which showed potency values at least half an order of magnitude apart could be considered to have different potencies. The relatively nonpotent compounds were those which had potencies an order of magnitude less than that of colchicine.

Toxicities. The toxicity of these compounds was examined in two ways. We experimentally determined the LD_{50} of a compound after a single (im) injection. A group of ten NIH general purpose mice (ca. 20 g) were used at each of several dose levels. Usually, at least five dose levels were used. The compound was introduced in an Emulphor EL-620 mixture.[33] One set of ten mice, injected (im) with the emulphor mixture alone, was used as a control for each compound. The LD_{50}, and its 95% confidence interval, was determined by probit analysis.[34] The surviving mice were counted 7 days after the single im injection. The time of death of the mice was variable. Generally, mice died after the 2nd or 3rd day with these colchicines. The LD_{50} values and their 95% confidence intervals were converted to micromoles per kilogram for "toxicity (LC)" in Table I.

A second indication of toxicity was obtained from the NCI P388 assay. In that assay, a series of nine injections (im) was given to mice over 9 days (one injection per day). The toxicity of the compound was determined on the 5th day. If one or more of the group of mice injected at a particular dose level died on, or by, the 5th day, we considered that dose level the toxic level for the drug. Alternatively, an average loss of weight of >3 g for the set of mice at a particular dose level was considered the toxic dose. Conversion to micromoles per kilogram gave the "toxicity (NCI)" values seen in Table I.

Tubulin Binding Assay. The binding of the colchicine analogues to microtubule protein was determined by measuring their ability to displace ^{3}H-labeled colchicine according to previously described procedures.[2] 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-cold solution containing 0.1 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 90%.

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(33) Emulphor EL-620 is a polyoxyethylated vegetable oil obtained from the GAF Corp., Linden, N.J.

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