Conformational Analysis of Colchicinoids Containing an Electron-Deficient Aromatic Ring on the B Ring[1]

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A dinitrophenyl substituent at the C-7 position of *N*-deacetylcolchicine and *N*-deacetylisocolchicine was shown to perturb the ^{1}H NMR and circular dichroic spectra of colchicinoid ring systems. The perturbations were explained by an interaction between the C-7 substituent and the A and/or C rings. Other aromatic substituents or large substituents located at the C-7 position (*J. Med. Chem.* **1983**, *26*, 1365) have not been reported to influence the *aS–aR* equilibrium of the phenyltropone ring junction. It was concluded that the sterically unfavored atropisomers were stabilized by the interaction of an electron-deficient ring with the more electron-rich A ring of the colchicinoid molecules.

Introduction

Colchicine (Figure 1) is the oldest and most studied member of the family of microtubule-inhibiting drugs.[2] Colchicine exerts its biological effects by binding to the soluble tubulin heterodimer. The colchicine–tubulin complex disrupts normal microtubule assembly and subsequently microtubule-mediated processes. Colchicine has thus been used as a tool to study microtubule events and is clinically useful in the treatment of gout.

The colchicine binding site on tubulin is selective for the conformational states of colchicinoids.[2c] The phenyltropone ring system must be in an *S* axial chirality (designated as *aS*) in order to bind to tubulin.[3] This conformational requirement is retained in other drugs that bind to tubulin at the colchicine site.[4] For example, steganacin and podophyllotoxin analogs with the aromatic rings in the same geometric arrangement as colchicine are potent inhibitors of [^{3}H]colchicine binding to tubulin.[5] In fact, the conformational requirement for drugs binding to the colchicine site is so specific that the tubulin binding activities in the steganacin series indicated that the original crystal structure of steganacin was incorrect. The correct orientation of the aromatic rings in steganacin was later defined after total synthesis.[6]

The structural features of colchicine that control the *aS–aR* conformational equilibrium are not entirely clear. It is known that deacetamidocolchicine, in which the C-7 carbon is a methylene, exists in solution as a racemic mixture of the two conformational isomers.[4] Most C-7 derivatives of colchicine exist exclusively in the *aS* conformation, which places the C-7 substituent in a pseudoequatorial orientation.[7] However, a recent photoaffinity-labeling derivative of colchicine, *N*-(4-azido-2-nitrophenyl)-*N*-deacetylcolchicine, was reported to exist in two conformational isomers.[8] Two different types of structures were proposed to account for the conformational isomers, and it was unclear what type(s) of stabilizing interactions would account for a second conformation of this molecule.

In this study, we have prepared derivatives of colchicine and isocolchicine in which an electron-deficient (dinitroaromatic) ring is covalently attached to the C-7 carbon (Figure 1). We have observed by chromatographic and spectroscopic means that these two compounds exist in at least two conformations in solution. We propose that the minor conformers of these two molecules are stabilized by association of the electron-deficient dinitroaromatic ring with the relatively electron-rich aromatic ring of colchicine. A similar interaction of the tropone C ring of colchicine and an aromatic amino acid may also be occurring in the colchicine binding site on tubulin.[2a,9]

Results

Evidence for Conformational Isomerism of DNP-DAC and DNP-IDAC. The products *N*-(2,4-dinitrophenyl)-*N*-deacetylcolchicine (DNP-DAC) and *N*-(2,4-dinitrophenyl)-*N*-deacetylisocolchicine (DNP-IDAC) (Figure 1) were synthesized by reaction of 2,4-dinitrofluorobenzene (FDNP) with *N*-deacetylcolchicine and *N*-deacetylisocolchicine, respectively. Thin-layer chromatographic analysis of the products revealed two distinct spots for each reaction. The two products observed by thin-layer chromatography for the reaction of FDNP with *N*-deacetylcolchicine could not be separated by radial chromatography in spite of the large differences in the R_f values. Two bands were clearly seen during chromatography, yet when the separated bands were analyzed by thin-layer chromatography the original two components were still present, which indicated that the two compounds were interconvertable. The two products were also shown to be interconvertable by HPLC. Each component of the

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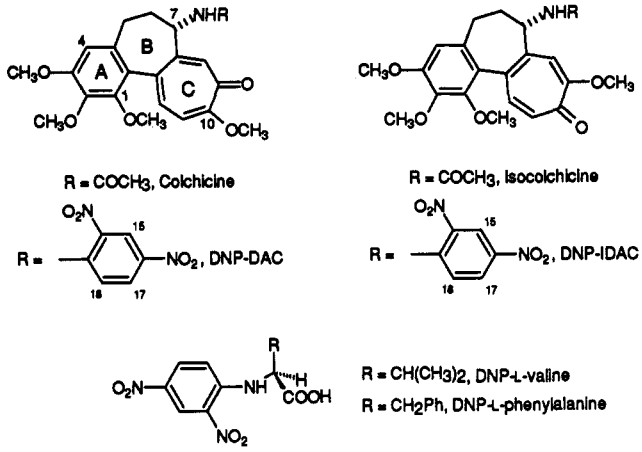

Figure 1. Structures of B-ring analogs of colchicine and isocolchicine, DNP-L-valine, and DNP-L-phenylalanine.

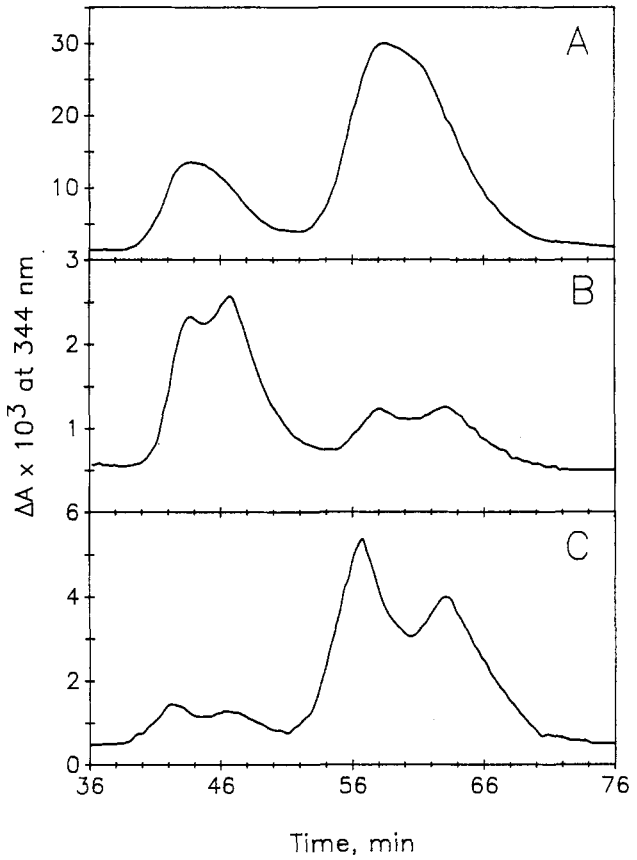

Figure 2. Separation of DNP-DAC atropisomers by reverse phase HPLC. (A) Injection of DNP-DAC into the HPLC resulted in two peaks with retention times of 43.6 and 58.2 min (relative areas were 23% and 77%, respectively). (B) The first peak from A was reinjected and resolved into two peaks with retention times of 44.8 and 60.8 min (relative areas were 71% and 29%, respectively). (C) The second peak from A was reinjected and resolved into two peaks with retention times of 44.8 and 61.0 min (relative areas were 13% and 87%, respectively). The mobile phase was 70:30 water–acetonitrile.

reaction mixture was isolated by HPLC, and the isolated components were reinjected into the HPLC (Figure 2). Each of the separate peaks again resolved into two components, and the individual components appeared to elute as two peaks. Thus, it appeared that the two products of the reaction of FDNP with *N*-deacetylcolchicine were conformational isomers.

Table I. ^{1}H NMR Spectral Data of Colchicine and Conformers of DNP-DAC[a]

		DNP-DAC conformers	
proton no.	colchicine[b]	major	minor
4	6.55 s	6.58 s	6.69 s
5	2.54 m	2.64 m[c]	*d*
	2.38 m	2.52 m	
6	2.38 m	2.52 m	
	2.01 m	2.13 m	
7	4.66 p (5.8, 11.0)[e] (5.5, 11.0)[e,f]	4.26 p (5.5, 11.2)[e]	4.60 t (6.6, ~0)[e]
8	7.69 s	7.34 s	7.52 s
11	6.93 d (11.0)	6.84 d (10.8)	6.82 d[d]
12	7.39 d (11.0)	7.32 d (10.7)	7.24 d (10.5)
1-OCH_3	3.67 s	3.85 s	3.16 s
2-OCH_3	3.95 s	3.92 s[g]	3.79 s[h]
3-OCH_3	3.92 s	3.89 s[g]	3.97 s
10-OCH_3	4.03 s	3.98 s	4.00 s[h]
NH	8.64 d (6.0)	8.79 d (5.6)[i]	7.86 d (6.3)[i]
$NHCOCH_3$	1.96 s		
15		9.10 d (2.5)	8.95 d (2.5)
17		8.01 dd (2.5, 9.4)	8.11 dd (2.5, 9.5)
18		6.17 d (9.4)	6.33 d (9.6)

[a] Determined at 360 MHz in $CDCl_3$, δ TMS = 0 ppm. Data are expressed as δ H, multiplicity, (*J*, Hz). [b] From Meksuriyen et al., 1988. [c] B ring protons were assigned from a ^{1}H–^{13}C HSC in acetone-d_6. [d] Signals of the minor conformer were obscured by the signals of the major conformer. [e] $J_{6,7}$. [f] From Brossi et al., 1988. [g] Signals may be assigned to the protons on either the C2-OCH_3 or the C3-OCH_3. [h] Signals may be assigned to the protons on either the C2-OCH_3 or the C10-OCH_3. [i] Assignment of signals was confirmed by a D_2O exchange experiment in DMSO-d_6.

The two products from the reaction of FDNP with *N*-deacetylisocolchicine were also found to interconvert. The conformers of DNP-IDAC, however, could be separated by radial chromatography and kept as dry solids at −20 °C for an indefinite period of time. Interconversion of the major conformer to the minor conformer in solution was rather slow, and the minor conformer was observed by thin-layer chromatography after approximately 1 day. The minor conformer was less stable, and the presence of the major conformer was detected after several hours.

^{1}H NMR Studies. The addition of the dinitrophenyl ring to the amino group of *N*-deacetylcolchicine and *N*-deacetylisocolchicine affected the ^{1}H NMR signals observed for the A and C ring protons as well as the signals due to the four methoxy groups (Tables I and II). Examination of the long-range coupling for each molecule aided in the assignment of the signals observed in the ^{1}H NMR spectra.

DNP-DAC. Assignment of the chemical shifts of DNP-DAC of the ^{1}H NMR signals for the two conformers of DNP-DAC was performed by long-range enhanced (LRE) COSY in $CDCl_3$ (Table I). The major conformer of DNP-DAC showed long-range coupling between the singlet at 3.85 ppm and the doublet at 7.32 ppm (C12-H), and the singlet was therefore assigned to the protons at the C1-OCH_3 position. The singlet at 3.98 ppm displayed long-range coupling with the doublet at 6.84 ppm (C11-H) and the signal at 3.98 ppm was assigned to the protons at the C10-OCH_3 position. Long-range coupling was observed for the singlet at 6.58 ppm (C4-H) with the singlet at 3.9 ppm. The exact chemical shift for the long-range coupling between the signal of the C4-H and the signal at 3.9 ppm could not be definitely determined. The signal at 3.9 ppm may be due to the protons of the C3-OCH_3 group. The chemical shifts of the two unassigned signals which correspond to the protons on the C2-OCH_3 or C3-OCH_3 (3.89 ppm and 3.92 ppm) were too close to be resolved.

Table II. ^{1}H NMR Spectral Data of Isocolchicine and Conformers of DNP-IDAC[a]

proton no.	isocolchicine[b]		DNP-IDAC conformers	
	major	minor	major	minor
4	6.56 s	6.64 s	6.65 s	6.73 s
5	2.51 m	2.58 m	2.68 m[c]	2.93 m
	2.36 m		2.54 m	2.68 m
6	2.30 m	2.70 m	2.68 m	2.68 m
	2.02 m	2.15 m	2.37 m	2.53 m
7	4.63 p (6.5, 12.5)[d]	5.00 t (7.5, ~0)[d]	4.39 p (5.9, 11.5)[d]	4.61 t (6.6, ~0)[d]
8	7.07 s	6.86 s	6.90 s	6.84 s
11	7.15 d (13.0)	7.16 d (13.0)	7.19 d (12.9)	7.14 d (12.9)
12	7.42 d (13.0)	7.33 d (13.0)	7.41 d (12.8)	7.29 d (12.9)
1-OCH_3	3.67 s	3.67 s	3.92 s	3.27 s
2-OCH_3	3.93 s	3.94 s	3.95 s	3.77 s
3-OCH_3	3.90 s	3.91 s	3.93 s	3.99 s
9-OCH_3	3.96 s	4.01 s	3.74 s	4.11 s
NH	6.33 d (7.0)	5.03 d (7.5)	8.80 d (5.5)	8.03 d (6.1)
$NHCOCH_3$	2.06 s	1.16 s		
15			9.12 d (2.4)	8.94 d (2.5)
17			8.11 dd (2.4, 9.4)	8.14 dd (2.5, 9.5)
18			6.38 d (9.6)	6.68 d (9.5)

[a] Determined at 360 MHz in $CDCl_3$, δ TMS = 0 ppm. Data are expressed as δ H, multiplicity (J, Hz). [b] From Gaffield et al., 1984. [c] B ring protons for the major conformer were assigned from a ^{1}H–^{13}C HSC in $CDCl_3$. [d] $J_{6,7}$.

Some long-range couplings were observed for the signals due to the minor conformer of DNP-DAC. Complete assignment of the signals due to the protons on the methoxy groups was not possible, as these signals were obscured by the signals of the major conformer. Long-range coupling was observed for the singlets at 3.97 and 6.69 ppm (C4-H), and the signal was assigned to the protons of the C3-OCH_3 group. The quadrapole-broadened doublet at 7.86 ppm showed long-range coupling to the triplet at 4.60 ppm (C7-H). The former signal was assigned to the hydrogen on the amine. Long-range coupling between a singlet at 3.16 ppm and a signal at near 7.2 ppm was observed and may be due to long-range coupling between the protons on the C1-OCH_3 group and the C12-H.

DNP-IDAC. Assignment of the chemical shifts for the two atropisomers of DNP-IDAC was easier than the analogous colchicine isomer since LRE COSY experiments could be performed on samples containing a single conformer. The assigned chemical shifts for the two atropisomers of DNP-IDAC and the chemical shifts previously determined for isocolchicine are listed in Table II. Complete assignment of the signals due to the protons of the methoxy groups was accomplished for the major conformer of DNP-IDAC. A singlet aromatic proton at 6.65 ppm (C4-H) displayed long-range coupling with a singlet at 3.93 ppm, and the signal was assigned to the C3-OCH_3 protons. Long-range coupling was observed between the doublet at 7.41 ppm (C12-H) and the singlet at 3.92 ppm and the latter signal was assigned to the C1-OCH_3 group. The singlet at 6.90 ppm (C8-H) showed long-range coupling with a singlet at 3.74 ppm. This upfield-shifted signal at 3.74 ppm was assigned to the C9-OCH_3 protons. By the process of elimination, the chemical shift of the C2-OCH_3 was assigned to the singlet at 3.95 ppm.

For the minor atropisomer of DNP-IDAC, a quadrapole-broadened doublet at 8.03 ppm was assigned to the amine proton, and this signal showed long-range coupling to the doublet at 6.68 ppm (C18-H). Two of the four signals due to the protons on the methoxy groups were assigned with assurance due to the existence of long-range coupling between the signals. The singlets at 6.84 and 6.73 ppm (C8-H and C4-H) displayed long-range coupling with the signals for the protons of the methoxy groups at 4.11 and 3.99 ppm, respectively. The signal at 4.11 ppm was assigned to the C9-OCH_3 protons, and the signal observed at 3.99 ppm was assigned to the C3-OCH_3 protons. A very weak long-range coupling may have existed between the doublet at 7.29 ppm (C12-H) and the singlet at 3.27 ppm. If this was indeed the case, then the signal at 3.27 ppm was probably due to the protons on the C1-OCH_3 group and therefore the signal at 3.77 ppm could be assigned to the protons on the C2-OCH_3.

Colchicinoid Ring Conformations of DNP-DAC and DNP-IDAC. The B ring proton resonances of the colchicinoid ring system constitute an ABCMX five-spin system. The coupling constants for these protons for colchicine and isocolchicine have been determined experimentally for the *aS* conformation and have been partially determined for the *aR* conformation of isocolchicine.[2c,10] The coupling pattern of the C-7 proton is diagnostic for the conformational state (*aR* or *aS*) of the ring system.

In the *aS* conformation of both colchicine and isocolchicine, the coupling constant for the C7-H and the *pro-R* proton on C-6 is 5.5–6.5 Hz, while the coupling constant for the C7-H with the *pro-S* proton on C-6 is 11–12.5 Hz.[2c,10] These data indicate that the C7-H is axially oriented, which is consistent with the *aS* conformation of the colchicinoid skeleton. The C7-H is also coupled to the proton on the adjacent nitrogen. In the 360-MHz ^{1}H NMR spectrum, the C7-H is observed as a pentet.

The coupling constants found for the C7-H of the major conformers of both DNP-DAC and DNP-IDAC are very similar to the coupling constants previously found for the C7-H of the *aS* conformation of colchicine and isocolchicine (Tables I and II). Morevoer, the entire B ring proton system for the major conformation of DNP-IDAC was simulated using the experimentally determined coupling constants for DNP-IDAC ($J_{6,7}$'s and $J_{7,NH}$) and $J_{5,6}$'s previously found for the *aS* conformation of isocolchicine (data not shown). It was not possible to analyze the high-field region of the spectrum for the major conformer of DNP-DAC due to the presence of the minor conformer in all spectra.

An atropisomeric transformation of the colchicinoid ring system to the *aR* conformation places the C7-H in a

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Table III. NOE Difference Spectroscopy of the Major Conformer of DNP-DAC[a]

irradiated δ, ppm, multiplicity, proton no.	enhancement δ, ppm, multiplicity, proton no.	% NOE
6.42 d, 18	2.78 m, 6	1.1
	4.42 p, 7	10.0
	7.21 s, 8	1.2
	8.20 dd, 17	2.9
6.85 s, 4	3.90 s, 3-OCH_3	1.4
8.20 dd, 17	6.42 d, 18	2.4
8.99 d, 15	2.78 m, 6	3.0

[a] Determined at 360 MHz in acetone-d_6, δ TMS = 0 ppm.

pseudoequatorial position. The *aR* conformation has been experimentally observed for natural (−)-isocolchicine but has not been seen in natural (−)-colchicine. In the ^1H NMR spectrum of the *aR* conformation of isocolchicine, the coupling constant of the C7-H with the C6-*pro-S* proton decreases to nearly zero as a result of changing the angle between the two hydrogens to ~90°, and the coupling constant between the C7-H and the C6-*pro-R* proton remains about 7.5 Hz. The C7-H appears as a triplet in the spectrum. In addition, the C7-H also undergoes a downfield shift upon atropisomerism, presumably as a result of removal of the C7-H from the A ring shielding cone.[10]

Although the *aR* conformation of (−)-colchicine has not been experimentally observed, the coupling constants for the B ring protons have been calculated.[2c] The calculated coupling constants for the B ring protons of the *aR* conformation of colchicine are similar to the experimentally determined coupling constants for the *aR* conformation of isocholchicine.

The chemical shift and coupling constants of the C7-H in the minor conformation of both DNP-DAC and DNP-IDAC are consistent with an *aR* conformation of the colchicinoid skeleton (Tables I and II). The ^1H NMR spectrum for the B ring protons of the minor conformer DNP-IDAC was also simulated using the experimentally determined coupling constants for DNP-IDAC ($J_{6,7}$'s and $J_{7,NH}$) and $J_{5,6}$'s previously found for the *aR* conformation of isocolchicine (data not shown). It is therefore reasonable to assign the major conformers of both DNP-DAC and DNP-IDAC as possessing the *aS* conformation and the minor conformers of the two molecules as existing in the *aR* conformation.

NOE Difference Spectroscopy of DNP-DAC and DNP-IDAC. NOE difference spectroscopy of the major conformations of DNP-DAC and DNP-IDAC was performed to gain insight into the preferred orientations of the dinitrophenyl ring with respect to the colchicinoid skeleton.

DNP-DAC. The irradiated signals, the percent NOE enhancement observed for the chemical shifts, and the assignment of these enhanced signals for DNP-DAC are listed in Table III. Irradiation of the signal at 6.85 ppm (C4-H) showed a small enhancement at 3.89 ppm (1.4%) which corresponds to the C3-OCH_3. This NOE enhancement confirmed the assignment of the C3-OCH_3 signal based on LRE COSY for DNP-DAC. Irradiation of the C18-H, located on the dinitrophenyl ring, produced weak enhancements at 2.78 and 7.21 ppm (C6-H, 1.1%, and C8-H, 1.2%, respectively), and a large enhancement of the quintet at 4.42 ppm (C7-H, 10.0%).

DNP-IDAC. The results obtained from NOE difference spectroscopy of the major conformer of DNP-IDAC are listed in Table IV. Irradiation of the C8-H at 6.90 ppm displayed two NOE enhancements, one at 8.80 ppm (NH, 3.6%) and the other at 3.74 ppm (C9-OCH_3, 3.7%). The latter enhancement correlated with the assignment of this signal to the C9-OCH_3 based on LRE COSY. Irradiation of the C18-H produced a large enhancement at 4.39 ppm (C7-H, 8.7%). This signal was confirmed by irradiating the C7-H at 4.39 ppm (C18-H, 17.9%). An additional NOE enhancement at 2.68 ppm (C6-H, 2.1%) was also observed upon irradiation of the C7-H at 4.39 ppm.

Table IV. NOE Difference Spectroscopy of the Major Conformer of DNP-IDAC[a]

irradiated δ ppm, multiplicity, proton no.	enhancement δ ppm, multiplicity, proton no.	% NOE
4.39 p, 7	6.38 d, 18	17.9
	2.68 m, 5 or 6	2.1
	8.80 d, NH	~1
6.38 d, 18	4.39 p, 7	8.7
	8.10 dd, 17	8.7
6.65 s, 4	2.68 m, 5	3.4
6.90 s, 8	3.74 s, 9-OCH_3	3.7
	8.80 d, NH	3.6
8.11 dd, 17	6.38 d, 18	1.6

[a] Determined at 360 MHz in $CDCl_3$, δ TMS = 0 ppm.

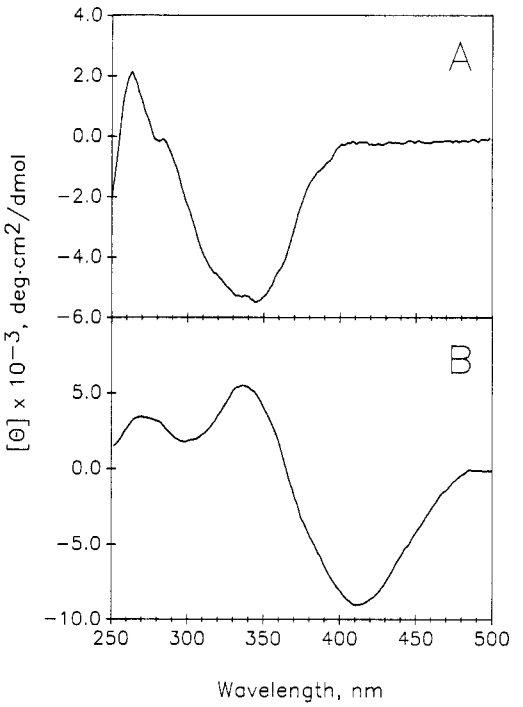

Figure 3. Near-UV CD spectra of (A) DNP-L-valine and (B) DNP-L-phenylalanine. The spectra were recorded in acetonitrile at ambient temperature and are presented in molar ellipticity.

Circular Dichroic Spectroscopy. The dinitrophenyl moiety absorbs in the same region of the spectrum as the tropone ring of colchicine. This substituent may also display CD bands in the near-UV region of the spectrum when bonded to the colchicinoid skeleton as a result of the chiral center at C-7. To observe the effect of a chiral center on the optical properties of the dinitrophenyl chromophore, the CD spectrum of DNP-L-valine was recorded in acetonitrile (Figure 3A). The CD spectrum of DNP-L-valine displayed a negative band centering at ~345 nm.

The effect of an aromatic ring in a chiral environment on the CD spectrum of the dinitrophenyl ring was also examined. The CD spectrum of DNP-L-phenylalanine

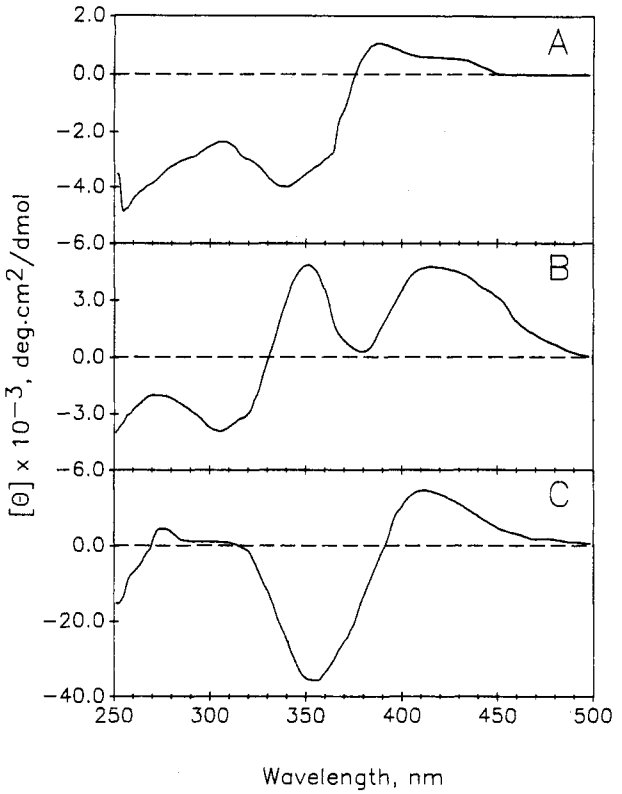

Figure 4. Near-UV CD spectra of (A) the major conformer of DNP-IDAC, (B) the minor conformer of DNP-IDAC, and (C) DNP-DAC. Spectra were recorded in acetonitrile at ambient temperature and are presented in molar ellipticity. The solution of the minor conformer was monitored carefully to ensure the purity of the sample. Note the difference in the *y*-axis scales in panels A–C.

(Figure 3B) in acetonitrile showed two small positive bands near ~270 and ~350 nm and a large negative band at ~410 nm.

The CD spectra of deacetylcolchicine and deacetylisocolchicine display strong negative bands in the region of 340 nm.[11] The effect of the addition of the dinitrophenyl ring to the two colchicinoid ring systems on the CD spectrum is shown in Figure 4. The CD spectra of the two conformations of DNP-IDAC were recorded separately. The major conformer of DNP-IDAC displayed two weak negative bands (340 and 390 nm; Figure 4A). The minor conformer showed three bands in the near-UV region of the CD spectrum (Figure 4B). Two positive bands were found in the lower energy region (352 and 414 nm), and a negative band was found at 312 nm.

The CD spectrum of the two conformers of DNP-DAC is shown in Figure 4C. A small positive band was observed at 388 nm while a large negative band was seen at 340 nm. The sign and molar ellipticity of the lower energy band was similar to the corresponding CD band of colchicine in solvent.[11]

Finally, the effect of tubulin binding on the near-UV CD spectrum of DNP-DAC was examined (Figure 5). The molar ellipticity of the negative CD band of DNP-DAC decreased by ~34% when DNP-DAC was bound to tubulin. Tubulin binding also induced an apparent red shift in this band.

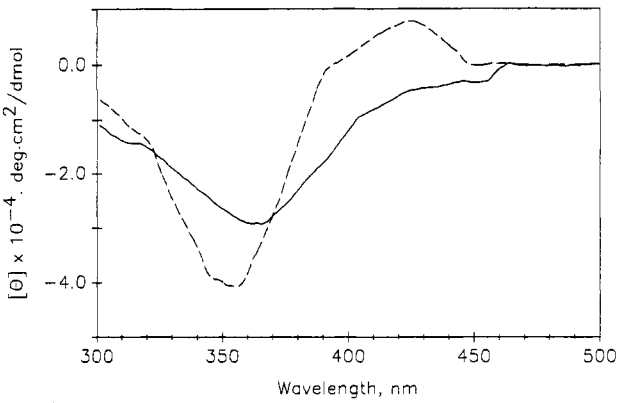

Figure 5. Near-UV CD spectra of DNP-DAC in the presence and absence of tubulin. The dashed curve is the spectrum of DNP-DAC in 5% DMSO-PMEG. The solid curve is the spectrum of DNP-DAC bound to tubulin after removal of unbound ligand. Spectra were recorded at ambient temperature and are presented in molar ellipticity.

Discussion

Atropisomerization of Colchicinoids. DNP-DAC and DNP-IDAC have been shown to exist in at least two conformations. The addition of the dinitrophenyl ring to *N*-deacetylcolchicine and *N*-deacetylisocolchicine appears to have affected the equilibrium of the two skeletal conformations, *aR* and *aS*, that can exist for colchicinoids. Previous studies of unnatural (+)-colchicine and natural (−)-colchicine have shown that the favored orientation of the B ring substituent is pseudoequatorial.[4] For (+)-colchicine to adopt a pseudoequatorial orientation of the B ring substituent, the axial asymmetry of the molecule must change from an *aS* to an *aR* configuration about the phenyltropone bond. The B ring substituent of the major conformers of DNP-DAC and DNP-IDAC is in a pseudoequatorial orientation. But the B ring substituent of the minor conformers for both DNP-DAC and DNP-IDAC is in a pseudoaxial orientation. Thus, it appears that the nature of the B ring substituent can influence the equilibrium of the two atropisomers, *aR* and *aS*, that may exist for colchicinoids.

If the steric properties of the substituent at the C-7 position are the driving force in the *aR*–*aS* equilibrium, then it would be anticipated that the two compounds prepared in this study, DNP-DAC and DNP-IDAC, would exist solely in the *aS* configuration. This is clearly not the case. Two conformational isomers that differ in chromatographic and spectroscopic properties were produced when the dinitrophenyl ring was added to each of the parent molecules.

Natural (−)-isocolchicine exists in two atropisomeric forms in apolar solutions.[10] The conformer in which the C-7 amide is pseudoequatorial (*aS*) predominates. The ratio of the two conformers indicates that the *aS* conformer is less than 3 kcal mol^{-1} more stable than the *aR* conformer.[12] There are few studies on the conformational properties of the colchicinoid ring system, and it is unclear why natural (−)-isocolchicine may be found in two atropisomeric forms in solution while only one conformation has been detected for natural (−)-colchicine. The differences between colchicinoid and isocolchicinoid ring systems may be the result of the intrinsic properties of the

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B ring system. The B ring of colchicine is a cycloheptene ring with two adjacent exocyclic double bonds while the B ring of isocolchicine resembles a cycloheptadiene ring system. It is known that additional double bonds and substituents on seven-membered rings can alter the availability of various conformations to the substituted ring systems.[13]

Furthermore, it has been predicted from molecular mechanic calculations that other conformations may be available to the colchicine and isocolchicine ring systems.[14] It was predicted that the relative populations of these other conformations of colchicinoids would be small in comparison to the two atropisomers that have been experimentally detected for isocolchicine. Therefore, these less favored conformations may be very difficult to detect.

Experimental support for the other conformations that were predicted to exist for colchicine and isocolchicine was found by studying the colchicine analogs DNP-DAC and DNP-IDAC. The two asymmetric peaks observed from the HPLC analysis of DNP-DAC (Figue 2) suggest that more than two conformations are available to this molecule. Additional conformations for DNP-IDAC were detected, albeit in small amounts, by thin-layer chromatography and ^{1}H NMR spectroscopy.

Possible Three-Dimensional Structures. The dinitrophenyl ring of DNP-DAC and DNP-IDAC should be able to rotate fairly freely about the N–C bond without large steric interactions between the dinitrophenyl ring and the colchicinoid and isocolchicinoid skeletons (as observed from Dreiding models). It is possible that this electron-deficient ring may be preferentially oriented with respect to the colchicinoid skeleton in such a way to gain stabilization by association with the relatively electron rich A and/or C ring(s). If so, the unusual chemical shifts observed in the ^{1}H NMR spectra may be explained by such an interaction. Assignment of signals due to most of the OCH_3 protons observed for DNP-DAC and DNP-IDAC were accomplished by analyzing long-range coupling constants. A combination of LRE COSY and NOE difference spectroscopy enabled us to propose the following possible three-dimensional structures for the conformers of DNP-DAC and DNP-IDAC in solution.

For the major conformer of DNP-DAC in $CDCl_3$, the signal for the C1-OCH_3 protons is shifted downfield in comparison to the signal for the C1-OCH_3 protons of colchicine (Table I). In $CDCl_3$, the C8-H in DNP-DAC is shifted upfield by 0.34 ppm with respect to the signal due to the C8 proton of colchicine. A small upfield shift is also observed for the signal of the OCH_3 protons at the C10 position. These unusual chemical shifts coupled with the NOE enhancements observed upon irradiating the C18-H (enhancements observed at the C7-H and C8-H; see Table III) may be explained by several possible orientations of the dinitrophenyl ring with respect to the colchicinoid frame. The ring may be oriented such that the plane of the dinitophenyl ring is approximately perpendicular to the colchicine skeleton and located either above or below the B ring portion of the colchicine frame. One possible orientation is shown in Figure 6A.

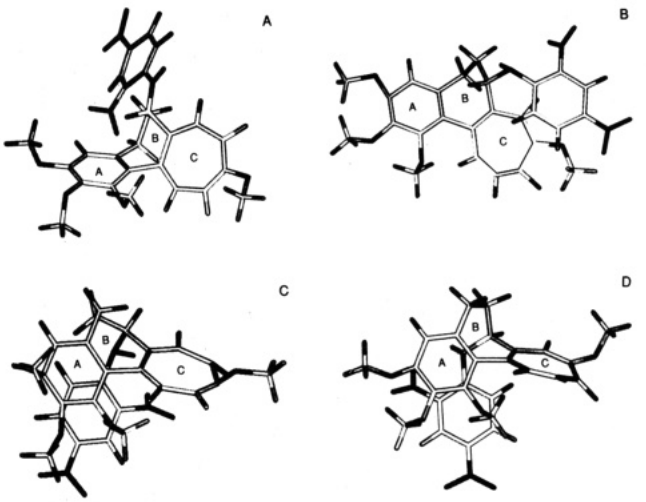

Figure 6. Possible three-dimensional solution structures of (A) the major conformer of DNP-DAC, (B) the major conformer of DNP-IDAC, (C) the minor conformer of DNP-DAC, and (D) the minor conformer of DNP-IDAC.

From the spectral data, the orientation of the dinitrophenyl ring of the major conformer of DNP-IDAC appears to be different from the analogous DNP-DAC conformer. The signals of the C8-H and the C9-OCH_3 were shifted upfield relative to the major conformer for isocolchicine (Table II). The signal due to the protons of the C1-OCH_3 was shifted downfield relative to the analogous signal observed for the major conformer of isocolchicine. NOE difference spectroscopy showed a strong interaction between the C18-H and the C7-H as well as an interaction between the NH and the C8-H (Table IV). To explain such a large upfield shift in the C9-OCH_3 signal and the observed NOE enhancements, the dinitrophenyl ring may be oriented such that the plane of the ring is approximately perpendicular to the methoxytropone C ring as depicted in Figure 6B. Either the dinitrophenyl ring current or the current from the nitro group may be responsible for the dramatic upfield shift observed for the signal of the C9-OCH_3 group. Such an orientation may stabilize the electron-deficient dinitrophenyl ring through an interaction with the C ring.

Possible three-dimensional structures for the two minor conformers of DNP-DAC and DNP-IDAC may be very similar. One of the striking features observed in the ^{1}H NMR spectra for both minor conformers is the large separation between the signals due to the protons on the methoxy groups and the dramatic upfield shifts observed for the signals due to the protons of the C1-OCH_3 and the C2-OCH_3 groups (Tables I and II, respectively). To explain such large shifts observed in the signals in this region of the spectra, the dinitrophenyl ring may be situated under the colchicine and isocolchicine frames (Figures 6C and 6D, respectively).

The proposed solution structures of the minor conformations of DNP-DAC and DNP-IDAC also provide a rationale for their existence. There must be a stabilizing force for the molecule to adopt the *aR* configuration which is sterically less favored for such a large substituent. The dinitrophenyl ring, by vitue of the two nitro groups, is quite electron deficient. The aromatic ring of colchicine, with three methoxy groups attached, is quite electron rich. The methoxytropone C ring also has a greater electron density relative to the dinitrophenyl ring. The ^{1}H NMR analyses indicated that the dinitrophenyl ring is perturbing

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certain A ring OCH_3 protons by ring current effects and also perhaps by the current effect from a nitro group. We therefore propose that the minor conformations of the dinitrophenyl derivatives of colchicine and isocolchicine may be stabilized by $\pi-\pi$ interactions between the two rings with differing electron densities.

Circular Dichroic Spectroscopy. The dintrophenyl chromophore is observed in the low-energy region of the absorption spectrum. The addition of this ring to the chiral center of the two parent molecules *N*-deacetylcolchicine and *N*-deacetylisocolchicine may affect the near-UV region of the CD spectrum. This possibility was examined by observing the effect of the added dinitrophenyl ring on the CD spectrum of two amino acids. L-Valine does not display a CD band in the low-energy region of the spectrum. The CD spectrum of DNP-L-valine, however, displayed a negative band with small molar ellipticity centering at ~345 nm. The effect of the dinitrophenyl chromophore on the aromatic amino acid L-phenylalanine was also examined. The derivative DNP-L-phenylalanine has two π-systems, and it appears that the π-system of L-phenylalanine perturbs the π-system of the dinitrophenyl ring. Thus, an interaction between the dinitrophenyl ring attached to a chiral center and another π-system can alter the low-energy region of the CD spectrum.

Evidence for an interaction of the π-systems of the dinitrophenyl ring and the colchicine ring system may also be observed by circular dichroic spectroscopy. It is known that the large negative CD band near 340 nm in colchicine and isocolchicine is related to the *aS* configuration about the phenyltropone ring junction.[4] The *aR* configuration of unnatural (+)-colchicine produces a large positive CD band near 340 nm. It has been previously shown that the energy and the magnitude of the near-UV CD band is affected by the aggregation state of the colchicinoid.[9] Specifically, dimerization of colchicine causes this band to decrease in magnitude and to red-shift. A similar effect is observed with tubulin binding, while solvents have little effect on this CD band.

The CD spectra of the two conformers of DNP-IDAC in solvents are quite different from the CD spectra observed for isocolchicinoids.[11] The small molar ellipticity of the two CD bands observed for the major conformer of DNP-IDAC is unusual for colchicinoids. The relative magnitude of the low-energy CD bands cannot be due to a simple sum of the CD spectra of two noninteracting chromophores (data not shown). Therefore, an interaction between the colchicine and the dinitrophenyl chromophores may be occurring to alter the sign of one of the CD bands (i.e., positive). The net result would be a smaller molar ellipticity of the observed CD band. The negative CD band at 340 nm of the major conformer may reflect an *aS* configuration of the phenyltropone bond. From the ^1H NMR analyses, we proposed that the dinitrophenyl ring and the methoxytropone C ring are oriented such that the electron-deficient ring may be stabilized by a π-stacking interaction. The relatively low intensities of the CD bands may reflect this type of an interaction. The minor conformer of DNP-IDAC also displays CD bands in the low-energy region of the spectrum (maxima at 352 and 414 nm); however, both bands are positive. The former band may be a reflection of the axial configuration (*aR*) about the phenyltropone bond of the minor conformer relative to the major conformer (*aS*). The positive bands observed for both conformers of DNP-IDAC around 400 nm may be due to the dinitrophenyl chromophore.

The CD spectrum of DNP-DAC differs from its isocolchicine isomer and resembles more closely the characteristic CD spectra observed for most colchicine analogs. The dinitrophenyl ring may be responsible for the small positive band observed in solvents around 400 nm. This observation is not surprising since the B-ring analog *N*-methyldemecolcine displays a small positive band at 395 nm. We have previously shown that the molar ellipticity of the low energy CD band of colchicine is affected slightly by the C-7 substituent.[15] In solvents, the molar ellipticity of the low energy CD band for aminocolchicinoids is slightly smaller than the molar ellipticity of colchicine in solvents. Aminocolchicinoids bound to tubulin, however, display a larger molar ellipticity when bound to tubulin than the colchicine–tubulin complex. The reduction of the molar ellipticity of the low-energy CD band of DNP-DAC bound tubulin was slightly less than the reduction that has been observed for other B-ring analogs of colchicine bound to tubulin. We proposed, based on the ^1H NMR spectrum, that the minor conformer of DNP-DAC exists with the dinitrophenyl ring oriented underneath the A and C rings. If the relatively small molar ellipticities observed for the atropisomers of DNP-IDAC are the result of an interaction between the π-systems of isocolchicine and the dinitrophenyl ring, then the CD spectrum of the minor conformer of DNP-DAC may be very weak and would therfore be obscured by the strong CD band observed for the major conformer of DNP-DAC.

Conclusions

The addition of an electron-deficient ring at the C-7 position has been shown to perturb both the ^1H NMR and circular dichroic spectra of colchicine and isocolchicine. The alterations in the spectra are similar to the perturbations observed in the ^1H NMR and circular dichroic spectra of colchicine upon dimerization. The changes observed in the spectra of colchicine upon dimerization have been proposed to be due to an intermolecular stacking interaction between colchicine molecules.[9] The dinitrophenyl ring at the C-7 position appears to be capable of an intramolecular π-stacking interaction between the dintrophenyl ring and the colchicine and isocolchicine frames.

The colchicine analogs DNP-DAC and DNP-IDAC are unusual in their chromatographic and spectroscopic properties. Most B-ring analogs of colchicine which contain a large substituent at the C-7*S* position exist in the *aS* configuration about the phenyltropone ring junction. So far, the only colchicine B-ring analog containing an aromatic moiety at the C-7 position that has been reported to exist in two conformational isomers is the photoaffinity label *N*-(4-azido-2-nitrophenyl)-*N*-deacetylcolchicine. This derivative is structurally very similar to DNP-DAC, and its B ring substituent is also quite electron deficient relative to the electron densities of the A and C rings. Thus, the minor conformations of DNP-DAC, DNP-IDAC, and possibly the photoaffinity label are most likely the result of an atropisomerization of the colchicinoid skeleton and are stabilized by the interaction of the B ring with substituent and the A and/or C rings.

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Experimental Section

Pipes (free acid), EGTA, $MgSO_4$, DTE, GTP (sodium salt, Type II-S), L-valine, and L-phenylalanine were purchased from Sigma. Acetone-d_6, $CDCl_3$, and DMSO-d_6 were purchased from Aldrich, and 2,4-dinitrofluorobenzene (FDNP) was purchased from Eastman Organic Chemicals. Colchicine was obtained from U. S. Biochemical Corp., and [^{3}H]colchicine was purchased form DuPont New England Nuclear Research Products. The other reagents and solvents were purchased from Fisher. Acetonitrile was HPLC grade, DMSO was spectrophotometric grade, and all other solvents were ACS certified.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM360 spectrometer interfaced to Aspect 3000 software. Me_4Si (0.3%) was used as an internal standard for ^{1}H NMR spectra, and $CDCl_3$ or acetone-d_6 was used as an internal standard for ^{13}C NMR spectra (δ 77.0 ppm and 30.1 ppm, respectively). Infrared spectra were measured in chloroform on a Nicolet 20SXC FTIR with a 660 data station. High- and low-resolution mass spectra (HRMS and LRMS, respectively) were recorded on a VG 70-70 spectrometer at an ionization voltage of 16 eV. Optical rotations were measured on a Perkin-Elmer Model 243B polarimeter. Melting points were measured on amorphous solids on a Sybron/Thermolyne melting point apparatus and are uncorrected. Scintillation spectrometry was performed on a Beckman LS 7500 scintillation spectrometer. Absorption spectra were recorded on a Hewlett-Packard Model 8451A diode array spectrometer.

Radial chromatography was performed on a Model 7945T Chromatotron (Harrison Research) using Merck silica gel PF-254 with $CaSO_4 \cdot {}^1/_2H_2O$. Silica gel plates, Merck F_{245} with a thickness of 0.25 mm, were employed for thin-layer chromatography, and Baker analyzed silica gel (60–200 mesh) was used for column chromatography. Separation of DNP-DAC conformers was performed on a Bio-Rad HPLC Gradient Module Series 400 connected to an ISCO V^4 variable-wavelength absorbance detector. A Bio-Rad Hi-Pore reversed-phase column RP-318 (250 mm × 4.6 mm) was employed.

N-Deacetylcolchicine and N-Deacetylisocolchicine. *N*-Deacetylcolchicine and *N*-deacetylisocolchicine were prepared according to a literature procedure.[16] The structure and purity of the products were confirmed by thin-layer chromatography, LRMS, and ^{1}H NMR spectroscopy.

Preparation of N-2,4-Dinitrophenyl Derivatives. *N*-2,4-Dinitrophenyl derivatives of deacetylcolchicine, deacetylisocolchicine, L-valine, and L-phenylanaline were prepared by reacting 2,4-dinitrofluorobenzene (FDNP) with the appropriate substrate.[17]

***N*-(2,4-Dinitrophenyl)-*N*-deacetylcolchicine (DNP-DAC).** The product consisted of two spots with R_f values of 0.34 and 0.46 (95:5 ethyl acetate–methanol). The overall yield was 45%. Two distinct bands were observed by radial chromatography; however, thin-layer chromatographic analysis of the fractions collected always showed two spots. The two spots were separated by HPLC on a C-18 column with a solvent mixture of 70:30 water–acetonitrile. Two peaks were observed with retention times of 43.6 and 58.2 min (relative areas 23% and 77%, respectively). Fractions of each peak were saved, and the solvent was evaporated. The separated peaks were reinjected into the HPLC, and the two peaks emerged. It appeared that each peak could be further resolved into two peaks under different solvent conditions. The retention times and areas (%) for the reinjected first peak were 44.8 (71%) and 60.8 min (29%). The retention times and areas were 44.8 (13%) and 61.0 min (87%) for the reinjected second peak. The two spots by thin-layer chromatography were confirmed to be atropisomers by ^{1}H NMR spectrsocopy and exist in a 5:1 ratio in $CDCl_3$: mp 128–130 °C; $[\alpha]^{22}_D$ +24.5–45.3 ° (*c* 0.53, EtOAc), mutarotation occurs over time and thus a single value can not be reported (time span 75 min); FTIR ($CHCl_3$) ν 3362.5, 3101.8, 2999.7, 2965.9, 2941.4, 2841.4, 1621.7, 1593.0, 1339.6, 1321.3 cm^{-1}; LRMS (*m/z*) M$^+$ 523 (524 also observed), 495, 449, 357, 328, 282; HRMS calcd for $C_{26}H_{25}O_9N_3$ was 523.1589, found 523.1647. **Major conformer:** ^{1}H NMR (acetone-d_6) δ 8.99 (1 H, d, J = 2.4 Hz, C15-H), 8.20 (1 H, dd, J = 2.5 and 9.1 Hz, C17-H), 8.83 (1 H, d, J = 5.6 Hz, NH), 7.21 (1 H, s, C8-H), 7.32 (1 H, d, J = 10.7 Hz, C12-H), 7.04 (1 H, d, J = 10.8 Hz, C11-H), 6.85 (1 H, s, C4-H), 6.42 (1 H, d, J = 9.3 Hz, C18-H), 4.42 (1 H, p, J = 5.5 and 11.2 Hz, C7-H), 3.94 (3 H, s, 10-OCH$_3$), 3.92 (3 H, s, 2-OCH$_3$ or 3-OCH$_3$), 3.89 (3 H, s, 2-OCH$_3$ or C3-OCH$_3$), 3.86 (3 H, s, 1-OCH$_3$), 2.78 (1 H, m, C5-H), 2.58 (2 H, m, C5-H and C6-H), 2.40 (1 H, m, C6-H); ^{13}C NMR (acetone-d_6) δ 179.0 (C9), 164.9 (C10), 154.6, 151.7, 147.6, 147.2, 142.4, 137.6, 136.5 (C12), 135.1, 134.9, 134.1, 131.8 (C8), 130.7 (C17), 125.6, 124.1 (C15), 116.2 (C18), 112.4 (C11), 108.5 (C4), 61.7 (C1-OCH$_3$), 61.0 (C3-OCH$_3$), 57.5 (C7 and C10-OCH$_3$), 56.2 (C2-OCH$_3$), 38.0 (C6), 30.1 (C5). **Minor conformer:** ^{1}H NMR (acetone-d_6) δ 8.27 (1 H, dd, C17-H), 7.88 (1 H, d, J = 6.2 Hz, NH), 7.51 (1 H, s, C8-H), 7.19 (1 H, d, C12-H), 7.02 (1 H, d, C11-H), 6.98 (1 H, s, C4-H), 5.00 (1 H, t, J = 6.6 Hz and ~0 Hz, C7-H), 3.99 (3 H, s, 10-OCH$_3$ or 2-OCH$_3$), 3.96 (3 H, s, 3-OCH$_3$), 3.76 (3 H, s, 2-OCH$_3$ or 10-OCH$_3$), 3.16 (3 H, s, 1-OCH$_3$); ^{13}C NMR (acetone-d_6) δ 138.4 (C8), 137.7 (C12), 136.5 (C11), 131.1 (C17), 115.5, 112.4, 109.0 (C4), 61.7 (C10-OCH$_3$), 61.0 (C3-OCH$_3$), 60.6 (C1-OCH$_3$), 57.5 (C2-OCH$_3$). Some of the ^{1}H and ^{13}C NMR signals of the minor conformation were obscured by the signals from the major conformation. Chemical shifts and coupling constants of the B ring protons were verified by decoupling experiments. Partial assignment of the ^{13}C NMR signals was from a ^{1}H–^{13}C heteronuclear shift correlation spectrum (HSC) in acetone-d_6.

***N*-(2,4-Dinitrophenyl)-*N*-deacetylisocolchicine (DNP-IDAC).** Two spots were readily observable by thin-layer chromatographic analysis (95:5 ethyl acetate–methanol) with R_f values of 0.53 and 0.26 (major conformer and minor conformer, respectively). The overall yield was 66%. The two coformers were easily separated by radial chromatography (95:5 ethyl acetate–methanol). The major conformer could remain in solution ($CDCl_3$) for ~4 days at room temperature before the other conformer was detectable by thin-layer chromatographic and ^{1}H NMR analyses. The major conformer in a solution containing the minor conformer was detectable within 24 h. The two conformers could be kept indefinitely as dry solids at −20 °C: mp 122–124 °C; $[\alpha]^{24}_D$ +8.1° for the major conformer (*c* 1.13 EtOAc) and $[\alpha]^{22}_D$ + 25.2° (*c* 1.03, $CDCl_3$) for the minor conformer (a ^{1}H NMR spectrum of the solution used for measuring the optical rotation of the minor conformer was taken just after the optical rotation measurement to ensure the purity of the minor conformer); FTIR ($CHCl_3$) ν 3364.0, 3005.6, 2935.2, 2856.0, 2829.6, 1619.9, 1594.9, 1339.9, 1321.2 cm^{-1}; LRMS (*m/z*) M$^+$ 523 (524 also observed), 495, 449, 357, 341, 328, 282; HRMS calcd for $C_{26}H_{25}O_9N_3$ 523.1589, found 523.1586. **Major conformer:** ^{13}C NMR ($CDCl_3$) δ 179.2 (C9), 164.0 (C10), 154.3, 150.7, 147.0, 141.1 (C12), 140.4, 137.3, 134.8, 134.5 (C11), 130.5 (C17), 124.5, 123.9 (C15), 115.0 (C18), 108.1 (C8), 107.4 (C4), 61.2 (C1-OCH$_3$), 61.1 (C2-OCH$_3$), 56.8 (C7), 56.0 (C9-OCH$_3$ and C3-OCH$_3$), 39.6 (C6), 29.7 (C5). **Minor conformer:** ^{13}C NMR ($CDCl_3$) δ 179.1, 163.6, 150.7, 147.0, 145.7, 142.2, 141.6, 136.1, 136.0, 134.7, 133.2, 130.4, 124.1, 115.2, 113.7, 108.0, 61.4, 60.5, 56.6, 56.5, 56.1, 40.9, 29.8. Chemical shifts and coupling constants of the B ring protons (Table II) were verified by decoupling experiments. Partial assignment of ^{13}C NMR signals for the major conformer was from a ^{1}H–^{13}C HSC spectrum in $CDCl_3$. Some of the ^{13}C NMR signals of quaternary carbons for the minor conformer were not detected.

***N*-(2,4-Dinitrophenyl)-L-valine (DNP-L-valine):** ^{1}H NMR ($CDCl_3$) δ 9.18 (1 H, d, J = 2.9 Hz), 8.93 (1 H, d, J = 7.7 Hz), 8.29 (1 H, dd, J = 2.7 and 9.6 Hz), 6.84 (1 H, d, J = 9.3 Hz), 4.22 (1 H, dd, J = 5.0 Hz), 2.45 (1 H, m), 1.18 (3 H, d, J = 6.9 Hz), 1.14 (3 H, d, J = 6.9 Hz).

***N*-(2,4-Dinitrophenyl)-L-phenylalanine (DNP-L-phenylalanine):** ^{1}H NMR ($CDCl_3$) δ 9.12 (1 H, d, J = 5.5 Hz), 8.87 (1 H, d, J = 7.4 Hz), 8.20 (1 H, dd, J = 2.6 and 9.5 Hz), 7.3 (5 H, m), 6.69 (1 H, d, J = 9.2 Hz), 4.62 (1 H, m), 3.44 (1 H, dd, J = 4.9 and 14.0 Hz), 3.26 (1 H, dd, J = 7.7 and 13.9 Hz).

NMR Studies. Colchicinoids (20–40 mg) were dissolved in a deuterated solvent ($CDCl_3$, acetone-d_6, or DMSO-d_6). To obtain an adequate amount of material for NMR experiments of the minor conformer of DNP-IDAC, the major conformer was refluxed in ethyl acetate for several hours, the solvent was evaporated, and the two conformers were separated by radial chromatography (95:5 ethyl acetate–methanol). Another set of

(17) Sanger, F. *Biochem. J.* **1945**, *39*, 507.

conformers was often detected in small amounts by thin-layer chromatography and ^1H NMR spectroscopy. The ratio of these conformers to the two conformers studied were extremely small, and thus these conformers were not examined.

For NOE difference spectroscopy measurements, the solutions were degassed in the NMR tube by seven cycles of the freeze–pump–thaw method and then closed under a nitrogen atmosphere.[18] Both NOE and control spectra were run under similar conditions except for the frequency of irradiation. The power level was chosen such that is was below saturation, and an irradiation time of 8 s was used. A 35-s delay time followed the 90° pulse. The long relaxation delay was chosen to ensure complete recovery of the equilibrium magnetization of all nuclei of interest.[19] A delay time of 0.1 s was used to ensure adequate time for frequency switching. Data sets of 32K covering a spectral width of 4000 Hz were acquired. Eight scans were taken for each irradiation frequency, and the cycle was repeated eight times with two additional scans included as a control. Each NOE difference experiment was run at least twice to ensure reproducibility of the NOE enhancement observed.

All long-range enhanced homonuclear shift correlation spectra (LRE COSY) were performed in $CDCl_3$ with a sweep width of 2520 Hz (2.5–9.5 ppm). To enhance the long-range effects D2, the parameter used to enhance small J was 0.08 s and a 90° pulse was used. Each LRE COSY was run twice.

Circular Dichroic Spectroscopy. Circular dichroic spectra were obtained on a JASCO Model J-20 ORD/UV-5 spectropolarimeter equipped with a Sproul Scientific SS15-2CD modification. CD spectra of DNP-L-valine (50.4 μM), DNP-L-phenylalanine (50.8 μM), DNP-DAC (53 μM), and the separated conformers of DNP-IDAC (59 and 51 μM for the major and minor conformers, respectively) were taken at ambient temperature (~23 °C) in acetonitrile. The purity of the solutions used for CD spectroscopy for the separated conformers of DNP-IDAC was checked by thin-layer chromatography during the experiment. At least three scans were made of each sample, and a base line was always recorded in duplicate. The data were digitized using Un-Plot-It (Silk Scientific Inc., Orem, UT) connected to an IBM PC-AT and stored in the form of ASCII files for subsequent manipulation. The concentrations of the solutions were determined by using an extinction coefficient calculated at the near-UV absorption maximum in acetonitrile. The extinction coefficients used were as follows: DNP-L-valine at 346 nm (1.66×10^4 M^{-1} cm^{-1}), DNP-L-phenylalanine at 348 nm (1.58×10^4 M^{-1} cm^{-1}), DNP-DAC at 346 nm (2.0×10^4 M^{-1} cm^{-1}), and DNP-IDAC at 342 and 346 nm for the major and minor conformers respectively (2.88×10^4 M^{-1} cm^{-1} and 2.74×10^4 M^{-1} cm^{-1}). The circular dichroic spectrum of DNP-DAC (20 μM) in the presence and absence of tubulin (10 μM) were recorded in 5% DMSO-PMEG (PMEG: 0.1 M Pipes, 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM GTP, pH 6.90 at 23.0 °C) at ambient temperature. The complex was subjected to gel filtration to remove excess ligand as previously described.[20] The concentrations of tubulin and DNP-DAC after removal of excess ligand were 10.0 μM.

Tubulin Purification and Protein Determination. Bovine brain tubulin, free of microtubule-associated proteins, was prepared by two cycles of assembly/disassembly followed by phosphocellulose chromatography and frozen in liquid nitrogen.[21] Tubulin obtained from this procedure is routinely >98% pure when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Prior to use, tubulin pellets were gently thawed, centrifuged at 800g for 10 min at 4 °C, and then desalted into PMEG buffer on 1 mL of Sephadex G-50 columns according to the method of Penefsky.[20] Tubulin concentrations were determined spectrophotometrically by the use of an extinction coefficient at 278 nm of 1.23 $(mg/mL)^{-1}$ in PMEG buffer.[22]

Inhibition of [^{3}H]Colchicine Binding. Quadruplicate solutions (70 μL) containing tubulin (5 μM), [^{3}H]colchicine (4 μM), and DNP-DAC (68.3 μM and 136.6 μM) in 14.3% DMSO-PMEG were incubated at 37 °C for 90 min prior to removal of excess ligand from each sample (60-μL aliquots) by rapid gel filtration by the method of Penefsky.[20] Samples containing tubulin (5 μM) and [^{3}H]colchicine (4 μM) in 14.3% DMSO-PMEG were used as a control. Scintillation fluid (1 mL) was added to each sample, and the samples were analyzed by scintillation spectrometry. The colchicine analog was found to inhibit [^{3}H]-colchicine binding to tubulin (68.3 μM DNP-DAC, 74% inhibition and 136.6 μM DNP-DAC, 82% inhibition).

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Supplementary Material Available: ^1H NMR spectra of DNP-DAC, DNP-IDAC, DNP-L-valine and DNP-L-phenylalanine (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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