Stereochemical variations on the colchicine motif. Part 2.[1] Unexpected tetracyclic isoxazole derivatives

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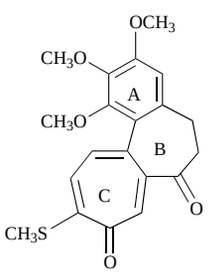
Attempts to expand the colchicine B-ring in 7-oxodeacetamidothiocolchicine 1 by a Beckmann-type rearrangement lead to unexpected tetracyclic isoxazole derivatives 2 and 3. The syntheses, crystal and solution structures, conformational interconversions and binding properties to tubulin are reported. The molecules exist as mixtures of two enantiomeric conformations due to hindered rotation around the A and C rings, which are twisted by dihedral angles of 62° (1) and 46° (2) in the crystal. Solid, solution and gas phase (according to MM2) structures are compared. Dynamic ^{1}H NMR analyses give the following thermodynamic parameters for the rotation around the A–C pivot bond: (1) $\Delta G^{\ddagger}_{381\,K} = 77.4$; (2) $\Delta G^{\ddagger}_{300\,K} = 60.7$; $\Delta H^{\ddagger} = 55.6 \pm 1.6$ kJ mol^{-1}; $\Delta S^{\ddagger} = -16.7 \pm 15$ J mol^{-1} K^{-1}; (3) $\Delta G^{\ddagger}_{298\,K} = 60.1$, $\Delta H^{\ddagger} = 59.9 \pm 2.0$ J mol^{-1} and $\Delta S^{\ddagger} = -0.7 \pm 15$ J mol^{-1} K^{-1}. The drugs 1 and 2 depolymerize microtubules by binding to tubulin according to both *in vitro* and *in vivo* studies, but 1 is considerably more active than 2. Compound 3 does not seem to bind notably to tubulin.

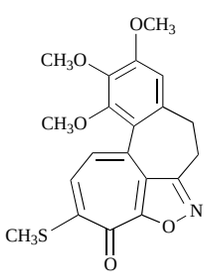
Introduction

The alkaloid colchicine, from *Colchicum autumnale*, exerts its major biological effect by binding to tubulin, the basic subunit component of microtubules. This process leads to depolymerization of the microtubules with concomitant mitotic arrest. The structure of the high-affinity binding site on one of the nonidentical subunits of tubulin, probably the β-subunit, is not known, but binding probably induces a conformational change in the protein, thereby inhibiting polymerization. Colchicine and its derivatives have been extensively studied from both chemical and biological perspectives.[2] We have been interested in the structural requirements of colchicinoids for binding to tubulin, in particular the conformation around the pivot bond joining the A and C rings.[3] The configuration around this bond is S_a and the angle between the A and C rings is close to 54° in colchicine and many derivatives. A suggestion that colchicine undergoes major conformational changes around this bond upon binding has been put forward[4] and has been rejected.[3,5,6]

This report deals with syntheses, X-ray diffraction structure determination, NMR spectroscopy, computation and competitive binding experiments of three new derivatives of colchicine: 7-oxodeacetamidothiocolchicine **1**, and its two isoxazolo-analogues, **2** and **3**. Two previously studied compounds were used as reference colchicine analogues: deacetamidocolchicine, DAAC and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone, MTC.



1



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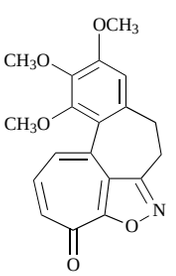

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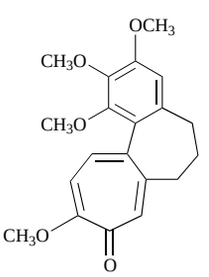
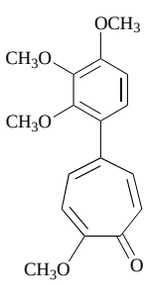


DAAC

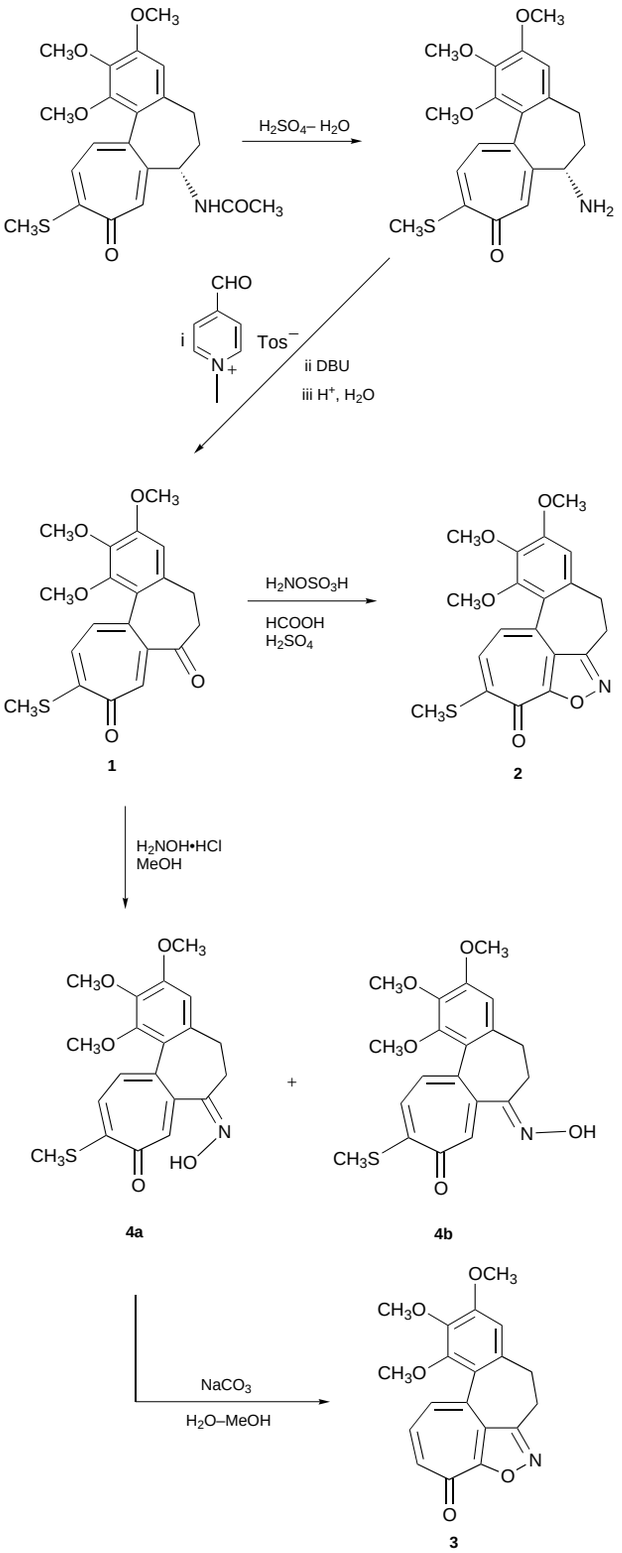


MTC

Results and discussion

Syntheses

The initial intention of this project was to prepare a B-ring expanded colchicine analogue *via* a Beckmann rearrangement of a keto derivative. Thiocolchicine could efficiently be transformed to **1** in two steps, hydrolysis and transamination, using a 4-formylpyridinum tosylate. In one of several attempts to expose the ketone to Beckmann conditions, treatment with hydroxylamine-*O*-sulfonic acid in formic acid with catalytic amounts of sulfuric acid, a slow reaction produced a new product identified as **2** in fair yield. The reaction sequence is shown in Scheme 1. The mechanism of the reaction leading to **2**, involving a formal oxidation, is not yet fully understood. Possibly, formic acid is involved, since the same reagent in the absence of formic acid produced the oximes **4a** and **4b**.[7]



Scheme 1

When one of the oximes, **4a** and **4b**, was treated with sodium carbonate in water–methanol at reflux temperature for 45 min, a new product was formed, which could be identified as **3** by its spectroscopic properties. Thus, the *syn*-structure for the isomer of the oxime was assigned.[8]

Crystal structures of 1 and 2

The compounds **1** and **2**, recrystallized from absolute ethanol, were examined by X-ray crystallography. Fig. 1 shows the molecular structures and the atomic numbering used. Bond distances and bond angles are given in Table 1. The crystals are racemates but all enantiomer-sensitive information, including

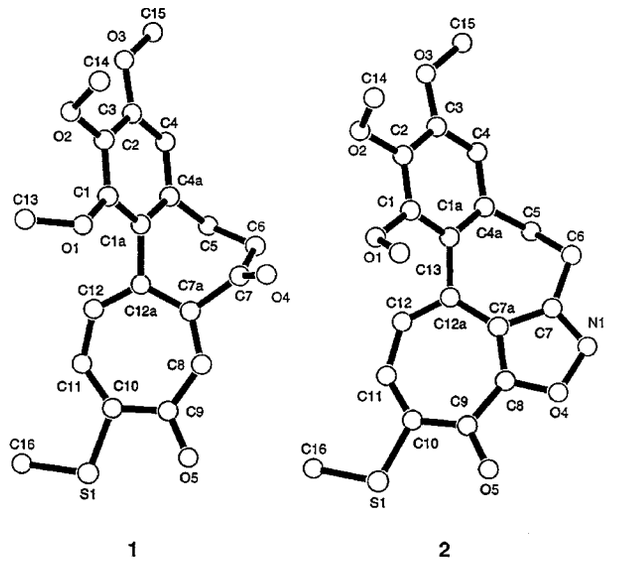


Fig. 1 Molecular structures of **1** and **2** in the crystals. Hydrogen atoms are omitted.

Fig. 1, is given for the atropisomer with *S*-biaryl configuration.[9] The molecules are arranged in the crystal lattices such that the polar carbonyl, methoxy and thiomethoxy groups approach each other. There are no very short intermolecular contacts; if the van der Waals radius of sulfur is assumed to be slightly shorter than the value 1.8 Å given by Bondi,[10] the S–S distance of 3.390(2) Å in **1** is also normal.

The C rings are fairly planar with average deviations from the least-squares plane of 0.035 and 0.064 Å for **1** and **2**. The conformations can be described as shallow boats with mirror planes through C9 and C11, respectively. The conformations of the B rings are close to the boat with a mirror plane through C5. For **1** the conformation is on the pseudorotation pathway towards a twist-boat with a twofold axis through C6. The B ring of **2** is somewhere between a C4a twist-boat and C5 boat.

The dihedral angles between planes A and C are 61.9 and 46.4°, for **1** and **2**, respectively. The high value for **1** is comparable with those of deacetylthiocolchicine hydrochloride dihydrate[11] and 7-oxodeacetamidocolchicine,[12] 60.1 and 61.6°. The unusually low value for **2** may be dependent on the extra strain introduced into the molecule by the isoxazole ring. The intramolecular O1–C12 distances are 3.030(5) and 2.814(9) Å, respectively. The corresponding O to H distances are 2.81(3) and 2.60(6) Å. Thus, there is contact between the 1-methoxy group and the tropone ring.

Both the A and C rings are tilted with respect to the pivot bond (C1a–C12a) as a result of the strain in the molecules; the angles between this bond and the normals to the least squares planes are for **1**: 95.5° to A and 89.4° to C, and for **2**: 96.7° to A and 78.1° to C.

The 3-methoxy and thiomethoxy groups are approximately in the planes of the corresponding rings in both molecules. The C3–C2–O2–C14 torsion angles are −76.1° and −76.2°. However, the C2–C1–O1–C13 torsion angle is +55.5° in **1** and −105° in **2**. Thus, the 1,2-dimethoxy groups are approximately antiparallel in **1** and parallel in **2**. The structure of **1** is very similar to that of the 10-methoxy analogue.[12] Actually, the crystals are isomorphous, which is evident after reduction of the cell of ref. 12 (a = 7.416, b = 10.498, c = 12.583 Å, α = 109.41, β = 105.60 and γ = 97.29°) and a shift of origin. These two similar molecules are the only examples so far known with the particular combination of orientations of the 1- and 2-methoxy groups, *i.e.* antiparallel with the same sign of the C2–C1–O1–C13 and the C1–C1a–C12a–C12 torsion angles. The parallel orientation of the 1,2-methoxy groups with opposite signs of the C2–C1–O1–C13 and the C1–C1a–C12a–C12

Table 1 Selected structural data for **1** and **2**

Bond distances/Å for **1**

S1	C10	1.754(4)	C2	C3	1.391(6)
S1	C16	1.796(6)	C3	C4	1.393(6)
O1	C1	1.356(4)	C4a	C4	1.396(6)
O1	C13	1.433(6)	C4a	C5	1.504(6)
O2	C2	1.370(5)	C5	C6	1.536(6)
O2	C14	1.435(5)	C6	C7	1.494(6)
O3	C3	1.359(5)	C7a	C7	1.522(5)
O3	C15	1.423(6)	C7a	C8	1.359(5)
O4	C7	1.209(4)	C7a	C12a	1.423(5)
O5	C9	1.268(4)	C8	C9	1.442(5)
C1a	C1	1.412(5)	C9	C10	1.456(5)
C1a	C4a	1.376(5)	C10	C11	1.358(5)
C1a	C12a	1.500(5)	C11	C12	1.415(5)
C1	C2	1.411(5)	C12a	C12	1.357(5)

Bond angles/° for **1**

C10	S1	C16	104.3(2)
C4a	C5	C6	110.4(4)
C1	O1	C13	119.4(3)
C5	C6	C7	113.6(3)
C2	O2	C14	113.5(4)
C3	O3	C15	117.0(4)
C8	C7a	C12a	130.4(3)
C6	C7	C7a	117.3(3)
C7a	C8	C9	132.2(4)
C8	C9	C10	122.8(3)
C9	C10	C11	127.6(3)
C10	C11	C12	131.4(3)
C7a	C12a	C12	124.3(3)
C11	C12	C12a	130.5(4)

Bond distances/Å for **2**

S1	C10	1.754(6)	C1a	C12a	1.499(8)
S1	C16	1.812(11)	C2	C3	1.394(8)
O1	C1	1.385(7)	C3	C4	1.372(9)
O1	C13	1.353(21)	C4	C4a	1.409(8)
O2	C2	1.371(7)	C4a	C5	1.510(10)
O2	C14	1.407(10)	C5	C6	1.525(10)
O3	C3	1.367(7)	C6	C7	1.499(9)
O3	C15	1.420(8)	C7	C7a	1.430(9)
O4	N1	1.397(7)	C7a	C8	1.378(8)
O4	C8	1.340(9)	C7a	C12a	1.416(9)
O5	C9	1.270(8)	C8	C9	1.481(9)
N1	C7	1.315(8)	C9	C10	1.421(11)
C1	C1a	1.395(9)	C10	C11	1.341(10)
C1	C2	1.385(8)	C11	C12	1.411(9)
C1a	C4a	1.400(8)	C12a	C12	1.364(10)

Bond angles/° for **2**

C4a	C5	C6	111.1(5)
C1	O1	C13	114(1)
C5	O6	C7	110.9(6)
C2	O2	C14	115.3(6)
N1	C7	C7a	111.6(5)
N1	O4	C8	108.7(5)
C6	C7	C7a	129.3(6)
O4	N1	C7	106.3(6)
C7	C7a	C8	102.9(6)
C8	C7a	C12a	129.5(6)
O4	C8	C7a	110.4(6)
C7a	C8	C9	133.5(7)
O5	C9	C8	115.9(8)
O5	C9	C10	123.8(7)
C8	C9	C10	120.2(6)
C9	C10	C11	127.9(6)
C10	C11	C12	132.7(8)
C7a	C12a	C12	121.6(6)
C11	C12	C12a	131.7(8)

torsion angles, as in **2**, seems to be the more common arrangement for the colchicine derivatives available in the Cambridge Structural Database.[13] For example, the structures of colchicine dihydrate,[14] deacetylthiocolchicine hydrochloride dihydrate[11] and thiocolchicine hexahydrate[15] show the same parallel methoxy groups.

Solution studies of 1, 2 and 3

The ^{1}H and ^{13}C NMR parameters of compounds **1**, **2** and **3** are presented in Table 2. All chemical shifts are close to those of analogous colchicine derivatives.[16] Assignments were derived with assistance from 2D NMR spectroscopy including COSY, NOESY and HETCOR experiments with the exception of C7 and C8 in **2** (δ 162.9 and 162.1). The NOESY spectra are in agreement with solution conformations concurring with the data for the crystal structures of **1** and **2** with one exception. There was no NOE between the 1- and 2-methoxy protons that would be expected for a *syn* conformation in **2**. However, this could have another cause and the question of the methoxy group conformation will be addressed in forthcoming work. A possible explanation is that the *syn* conformation found in the crystal is due to crystal packing properties, and that the most stable conformation of the 1- and 2-methoxy groups (δ 3.48 and 3.90) is perpendicular and *anti*, as is also observed in the crystal for many other colchicine analogues. The correlations between the 3-methoxy protons (δ 3.94) and the ring 4-proton (δ 6.61) and between the methylthio protons (δ 2.50) and the 11-proton (δ 7.23) indicate that the 3-methoxy and 10-methylthio groups spend considerable time in the planes of the A and C rings, respectively. A correlation between H(5_1) and H(4) in **1** indicates that H(5_1) lies in the A ring plane. Full assignment of the other B ring protons follows from the coupling constants. The NOESY experiments, however, do not give any information about the most important conformational feature, the torsional angle around the pivot bond.

Dynamic NMR spectroscopy

According to the results described above all the compounds **1**–**3** exist in conformations in which the A and C rings are twisted with respect to each other. Thus these molecules are chiral and exist in two enantiomeric atropisomers. We have shown that in the case of DAAC the two enantiomers can be resolved and that the barrier to rotation around the A–C-pivot bond is 92.5 kJ mol^{-1}.[2] Furthermore, only the enantiomer with the same helicity as natural colchicine binds to tubulin. We were not able to resolve either of the compounds **1**–**3** to enantiomers by chromatography.

The rotational barriers in atropisomeric biphenyls and bridged biphenyls have been extensively studied by dynamic NMR spectroscopy,[17] and the effects of the sizes of *ortho* substituents have been settled. In the colchicinoids one of the rings is a seven-membered ring which considerably increases the steric requirements in the transition state of the rotation. Such experiments showed that the barriers were significantly lower for **1**–**3** than in DAAC. The results of a dynamic NMR analysis of **2** in [$^{2}H_8$]toluene + 20% $CDCl_3$ are presented in Fig. 2. The thermodynamic parameters are $\Delta G^{\ddagger}_{300\,K} = 60.7$; $\Delta H^{\ddagger} = 55.6 \pm 1.6$ kJ mol^{-1}; $\Delta S^{\ddagger} = -16.7 \pm 15$ J mol^{-1} K^{-1}. The corresponding values for **3** in the same solvent are: $\Delta G^{\ddagger}_{300\,K} = 60.1$, $\Delta H^{\ddagger} = 59.9 \pm 2.0$ kJ mol^{-1} and $\Delta S^{\ddagger} = -0.7 \pm 15$ J mol^{-1} K^{-1}. Compound **1** could only be studied in a more limited temperature interval in [$^{2}H_2$]tetrachloroethane, and only the free energy of activation was evaluated; $\Delta G^{\ddagger}_{381\,K} = 77.4$ kJ mol^{-1}. Apparently, neither of the compounds have high enough barriers for resolution at moderate temperatures.

As expected the barrier decreases markedly in **2** and **3** due to the strain imposed by the isoxazole annelation. Less obvious is the comparatively low barrier in **1**, *ca.* 15 kJ mol^{-1} lower than in colchicine and DAAC, indicating considerable ground state strain.

Molecular mechanics and *ab initio* computations

A conformational analysis of **1**–**3** by the molecular mechanics program MM2 provides valuable structural information. Some structural parameters from MM2 and X-ray analyses are given in Table 3. The most definitive conformational attribute is the

dihedral angle around the pivot bond, being close to 54° in colchicine and in analogues with an unchanged ring structure. The force field, MM2(85) and later versions, reproduces satisfactorily the dihedral angle and other important structural features of colchicinoids.[2] The calculated values for this angle are 57° for **1** and 51° for **2** and **3**. An indirect measure of the twist angle is the puckering of the B ring and consequently also the torsional angle around the C5–C6 bond. Since this angle is connected to the vicinal coupling constants between the C5 and C6 protons, a comparison of experimental values with those calculated from the MM2 structures also allows comparison with solution geometries. The results are shown in Table 4. Good agreement is observed and gives credence to the use of MM2 geometries where experimental results are lacking.

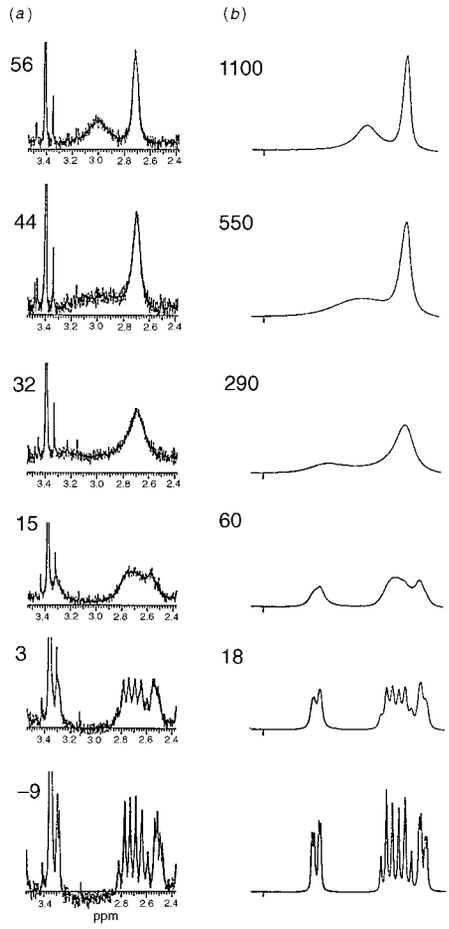


Fig. 2 Experimental (left) and DNMR5 calculated (right) ^{1}H NMR spectra at the temperatures indicated, in °C. Rate constants in s^{-1} are given above the calculated spectra. The signal at δ 3.3 is partially hidden under one methoxy signal.

The puckering of the C ring differs significantly in colchicine analogues. The tropolone ring is rather floppy due to the ring strain in the planar seven-membered ring,[18] and thus is susceptible to considerable departures from an essentially planar structure as a result of intramolecular interactions and, maybe, crystal packing effects or interactions with tubulin. Quantum chemical computations of methoxytropone give variable results as to the planarity of the ring. Semiempirical methods such as AM1 propose a nonplanar minimum energy structure, whereas a planar ring is obtained by *ab initio* calculations. At the RHF/6-31G* level the planar structure is a transition state to an out-of-plane vibration (imaginary frequency 25.5 cm^{-1}), but this low-frequency transition state is probably without significance, and the molecule assumes an essentially planar, shallow, minimum energy structure. The annelated isoxazole ring in **2** and **3** tends to increase puckering of the C ring compared with **1** according to MM2. This is in agreement with the crystal structure of **2**. In general, however, there is considerable variation in C ring puckering in crystals. One reason for this seems to be the floppyness of the ring, which enables comparatively large variations due to small effects, originating both in the substitution pattern and crystal packing. Furthermore, as was shown earlier the force field has to be finely tuned to reproduce the balance between conjugation and angle strain.[3]

The barriers to rotation around the pivot bond were calculated using the torsional driver technique, giving the values 79.9 and 58.6 kJ mol^{-1} for **1** and **2**, respectively, in good agreement with experiment.

Finally, the conformations of the methoxy groups were considered. The most stable conformation has the 1- and 2-

Table 2 ^{1}H and ^{13}C NMR data for **1–3**

	δ_H				δ_C		
	1	**2**[a]	**3**[a]		**1**	**2**	**3**
CH_3O (1)	3.57	3.48	3.54	C1	152.4	153.4	153.5
CH_3O (2)	3.88	3.90	3.91	C1a	125.1	124.0	123.9
CH_3O (3)	3.89	3.94	3.96	C2	142.0	142.5	142.4
H(4)	6.55	6.61	6.62	C3	154.4	154.4	154.7
$H(5_1)$	2.70	2.51	2.18	C4	107.5	108.2	108.0
$H(5_2)$	3.14	2.77	2.38	C4a	135.9	137.3	137.3
$H(6_1)$	2.85	2.68	2.33	C5	29.8	33.4	33.2
$H(6_2)$	2.95	3.32	3.00	C6	47.9	28.4	28.2
H(8)	6.96	—	—	C7	206.0	162.9[c]	163.3
CH_3S (10)	2.46	2.50	7.19[b]	C7a	150.2	121.6	121.1
H(11)	7.08	7.23	7.47	C8	130.5	162.1[c]	167.2
H(12)	7.23	7.37	7.27	C9	182.8	171.5	175.4
				C10	160.6	151.9	136.0
Coupling constants/Hz				C11	126.8	128.0	137.7
				C12	136.3	132.7	133.8
$H(5_1)$–$H(5_2)$	13.5	13	13	C12a	134.2	129.5	134.8
$H(5_1)$–$H(6_1)$	4.9	1	2	CH_3O (1)	61.7	61.9	61.9
$H(5_1)$–$H(6_2)$	2.3	4.6	3	CH_3O (2)	61.6	61.6	61.5
$H(5_2)$–$H(6_1)$	13.3	13	12	CH_3O (3)	56.5	56.5	56.5
$H(5_2)$–$H(6_2)$	5.4	1	2	CH_3S (10)	15.7	15.4	—
$H(6_1)$–$H(6_2)$	16.4	15	15				
H(11)–H(12)	10.6	10.5	9.4				
H(10)–H(11)	—	—	12.0				

[a] The chemical shifts and coupling constants for the 5- and 6-protons are evaluated from the low-temperature analysis. [b] Refers to H(10). [c] Assignments may be reversed.

Table 3 Torsional angles for some colchicinoids determined by X-ray crystallography and MM2 calculations (within parentheses)

Torsional angles	**1**	**2**	**3**	DAAC	MTC
C(4a)–C(1a)–C(12a)–C(7a)	59.5 (56.8)	48.7 (50.6)	— (51.3)	— (53.6)	57.4 (55.3)
C(12)–C(12a)–C(1a)–C(1)	60.1 (57.7)	44.7 (52.5)	— (52.1)	— (60.6)	(56.7)
C(13)–O(1)–C(1)–C(2)	55.5 (−98.9)	−105 (−98.2)	— (−98.3)	— (−98.5)	(−98.3)
C(14)–O(2)–C(2)–C(3)	−76.1 (97.5)	−76.2 (97.6)	— (97.5)	— (97.5)	(97.3)
C(15)–O(3)–C(3)–C(4)	−1.5 (9.0)	−6.3 (8.7)	— (9.9)	— (11.3)	(10.9)
C(11)–C(10)–X[a](1)–C(16)	3.7 (−0.1)	10.9 (0.1)	—	— (0.4)	(0.2)
Σ torsions in the B ring	296 (290)	252 (249)	— (251)	— (249)	—
Σ torsions in the C ring	32 (17)	62 (105)	— (73)	— (176)	(9)[b]

[a] S or O. [b] The value cannot be extracted from ref. 37, and the 3D coordinates are not available from the Cambridge Structural Database.

Table 4 Vicinal coupling constants for C(5)–C(6) protons from experiment and calculations on MM2 structures[38]

	1		**2**		**3**	
Dihedral angle	exp.	MM2	exp.	MM2	exp.	MM2
$H(5_1)$–$H(6_1)$	4.9	4.9	~1	2.8	2	2.7
$H(5_1)$–$H(6_2)$	2.3	3.5	4.6	6.0	4	6.0
$H(5_2)$–$H(6_1)$	13.3	12.7	13.0	12.8	13	12.8
$H(5_2)$–$H(6_2)$	5.4	4.9	~1	2.9	2	2.8

Table 5 *In vivo* depolymerization of microtubules by indirect immunofluorescence experiments with **1** and **2**[21]

	Concentration/nM at which microtubule networks retract	
Compound	Minor depolymerization	Complete depolymerization
Colchicine	64	250
Thiocolchicine	25	64
DAAC	10	50
MTC	64	250
1	16	64
2	64	250

methoxy groups approximately perpendicular to the aromatic ring and antiparallel to each other such that the methyl in the 1-substituent points away from the hydrogen on C12 in the tropolone ring. The parallel conformation, which is found in **2** and other structures in the crystal,[19,20] is calculated to be a local minimum 4.6–5.4 kJ mol^{-1} higher in energy. The ability of the force field to account for the lone pair interactions of the oxygen atoms could be questioned. In these calculations, no significant difference was obtained with and without inclusion of the lone pairs. The 3-methoxy group is more flexible and is essentially coplanar with the ring.

Binding to tubulin and depolymerization of microtubules

We have studied the depolymerization of microtubules by **1** and **2** by *in vivo* experiments.[21] The microtubule networks of cloned human prostate cells (DU 145) were observed by an indirect immunofluorescence technique before and after addition of various concentrations of the drug. The experimental details have been reported earlier.[22] Both **1** and **2** showed microtubule depolymerization, but at different drug concentrations. Some results are shown in Table 5. It turns out that, judging from this test, **1** belongs to the most powerful colchicinoids. Compound **3** was not studied in this test.

Many colchicine analogues show a strongly enhanced fluorescence upon binding to tubulin, a property which has been extensively used to study the kinetics of the binding process.[23] The compounds **1**–**3** did not show significant fluorescence enhancement upon mixing with tubulin. No significant fluorescence increase upon binding to tubulin has been reported for three other thiocolchicine analogues.[6] Compound **2**, however, showed a weak fluorescence when dissolved in the buffer solution.

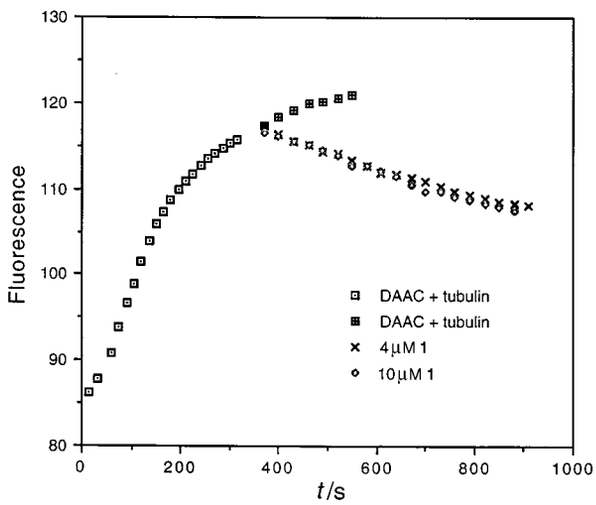


Fig. 3 Competition fluorescence development for the binding of DAAC with tubulin and subsequent addition of **1** at various concentrations

The tubulin-binding properties were instead demonstrated by competition binding experiments with other analogues, which exhibit fluorescence. Thus, the increase of fluorescence was followed when tubulin was incubated with an analogue and, after development until near equilibrium value, the decrease was followed by addition of the respective drug **1**–**3**. DAAC and MTC were chosen as competitors since they both give fluorescence upon binding to tubulin but bind with different rates.[3,23g] Fig. 3 shows such a competition experiment with DAAC (2 μM), tubulin (2 μM) and **1** (4 and 10 μM, respectively). The time-dependent decrease in fluorescence, after addition of **1**, was a slow process, nearly independent of the concentration of **1**. Reversed order, *i.e.* **1**, tubulin and DAAC, gave no significant change in the fluorescence in either step. The fact that there was no significant difference between 4 and 10 μM solutions of **1**, indicates that this drug binds strongly and practically irreversibly to tubulin in agreement with the immunofluorescence assay. It is also possible to evaluate an approximate off-rate constant for DAAC as 1.6×10^{-4} s^{-1} at 21 °C, a value in good agreement with earlier findings.[23g] The other analogues, **2** and **3**, did not show the corresponding decrease in competition with DAAC.

In order to estimate the binding properties of **2** and **3**, competition was studied with the MTC–tubulin complex, which is much weaker than the DAAC–tubulin complex. The binding of MTC was too fast to follow accurately with our technique, but the effects of addition of **1** and **2** after formation of the MTC–tubulin complex were similar to those with DAAC and **1**, whereas **3** did not show any significant change in fluorescence. The results are shown in Fig. 4.

Judging from these experiments **1** and **2** depolymerize microtubules by binding to the colchicine binding site in tubulin. The former compound is more potent according to both criteria.

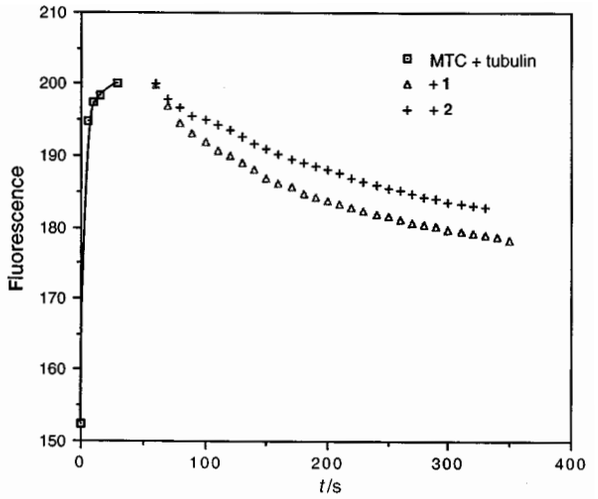


Fig. 4 Competition fluorescence development for the binding of MTC (2 μM) with tubulin (2 μM) and subsequent addition of **1** or **2** (4 μM)

Compound **3** does not show any sign of binding. The effect of variation of the 10-substituent has been studied including an unsubstituted analogue and a rather large tolerance to substitution in this position was found.[24] The unsubstituted derivative was active.

Conclusions

Three new colchicine derivatives have been synthesized and characterized. All three compounds are atropisomers and exist as racemates, and rotation around the pivot bond is rapid at room temperature, with rotational barriers in the range 60–77 kJ mol^{-1}. The dihedral angles between planes A and C are 61.9 and 46.4°, for **1** and **2**, respectively; two rather extreme values for colchicinoids possessing an intact ring skeleton. Solution studies and molecular mechanics computations give structures which agree with crystal structures with the exception of the orientation of the 1- and 2-methoxy groups for **2**. *In vivo* and *in vitro* experiments indicate that the compounds **1** and **2** bind to the colchicine binding site of tubulin, but **1** is considerably more active than **2**. This is consistent with earlier findings that colchicine analogues with small values of the A–C dihedral angle are less active.[6] Compound **3** does not seem to bind notably. A quantitative analysis of the tubulin binding properties of these and other colchicine analogues is in progress.

Experimental

Syntheses

7-Oxodeacetamidothiocolchicine, 1. To deacetylthiocolchicine (600 mg, 1.55 mmol) in a mixture of dichloromethane and dimethylformamide (DMF) (3:1, 48 ml) was added 4-formyl-1-methylpyridinium toluene-*p*-sulfonate (680 mg, 2.3 mmol). The resulting mixture was refluxed for 3 h. The reaction mixture was cooled in an ice–water bath and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (800 mg, 5.2 mmol) was added dropwise with stirring to afford a deep-purple solution. After 20 min oxalic acid (60 ml, 5%) was added to the vigorously stirred solution. The stirring was continued for 90 min at room temp. The aqueous and organic phases were separated and the water phase was extracted twice with dichloromethane. The combined organic phases were dried ($MgSO_4$) and concentrated to a yellow solid. Recrystallization from ethanol (96%) afforded yellow crystals as fine needles. Yield 67%; mp 228–229 °C; ν/cm^{-1} (film): 2938, 1702, 1601, 1562, 1488, 1344, 1095, 1025, 954 and 891; NMR see Table 2; MS: $C_{20}H_{20}O_5S$, calc. 372.1031; found 372.1033.

3-Methylthio-10,11,12-trimethoxy-7,8-dihydro-4*H*-benzo-[1,2]heptaleno[5,6-*cd*]isoxazol-4-one, 2. To 7-oxodeacetamidothiocolchicine **1** (137 mg, 0.37 mmol) in 97% formic acid (9 ml) was added a solution of hydroxylamine-*O*-sulfonic acid (100 mg) in formic acid (12 ml). A few drops of sulfuric acid were added and the solution was refluxed for 2 d. During this time hydroxylamine-*O*-sulfonic acid (300 mg) was added in portions. The reaction was monitored with TLC (dichloromethane–acetone, 9:1). The reaction mixture was poured on ice–saturated $NaHCO_3$ when no further reaction could be monitored. Chloroform was added and the phases were separated and the water phase was extracted twice with chloroform. The combined organic phases were washed once with water, dried ($MgSO_4$) and concentrated to a yellow solid. Flash chromatography (dichloromethane–acetone, 20:1) gave yellow crystals which could be recrystallized from ethanol (96%) to afford yellow crystals. Yield 35–41%; mp 226–227 °C; ν/cm^{-1} (film): 2940, 1605, 1574, 1491, 1465, 1343, 1315, 1105, 1016 and 932; NMR see Table 2; MS: $C_{20}H_{19}NO_5S$, calc. 385.0984; found 385.0984.

7-Oxodeacetamidothiocolchicine oximes, 4a and 4b. 7-Oxodeacetamidothiocolchicine (267 mg, 0.72 mmol) was partly dissolved in 92% methanol (14 ml). Hydroxylamine hydrochloride (56 mg, 0.81 mmol) and sodium carbonate (22 mg, 0.27 mmol) were added and the mixture was refluxed. After 24 h water and ethyl acetate were added and the aqueous and organic phases were separated. The water phase was extracted three times with ethyl acetate and the combined organic phases were dried (Na_2SO_4) and concentrated, yielding a yellow solid as a mixture of isomers. The isomers were separated by flash chromatography (ethyl acetate–heptane) yielding **4b** as the first eluted isomer and **4a** as the last eluted isomer. The oximes were recrystallized from ethanol (96%) to afford yellow crystals. The oximes had the following physical properties: **4a**: Yield 22%; mp 237–238 °C (decomp.); ν/cm^{-1} (film): 3400, 2938, 1601, 1563, 1488, 1345, 1322, 1135, 1028 and 750; $\delta_H(CDCl_3)$ 7.32 (1H, d, *J* 10.5), 7.10 (1H, d, *J* 10.5), 7.0 (1H, s), 6.68 (1H, s), 6.54 (1H, s), 3.90 (3H, s), 3.89 (3H, s), 3.58 (3H, s), 2.9–2.6 (4H, m); $\delta_C(CDCl_3)$ 181.8, 159.5, 158.6, 153.5, 151.6, 143.4, 141.5, 136.2, 135.4, 134.6, 132.1, 126.5, 126.2, 107.6, 61.9, 61.3, 56.0, 37.5, 29.7, 15.3; MS: $C_{20}H_{21}NO_5S$, calc. 387.1140; found 387.1138. **4b**: Yield 63%; mp 236–238 °C (decomp.); ν/cm^{-1} (film): 3350, 2934, 1596, 1524, 1489, 1456, 1346, 1137, 1097 and 989; $\delta_H(CDCl_3)$ 9.5 (1H, s), 7.34 (1H, s), 7.23 (1H, d, *J* 10.5), 7.10 (1H, d, *J* 10.6), 6.54 (1H, s), 3.90 (1H, s), 3.89 (1H, s), 3.54 (1H, s), 3.5–3.4 (2H, m), 2.8–2.5 (2H, m); $\delta_C(CDCl_3)$ 182.7, 161.6, 159.2, 154.0, 152.0, 145.6, 141.7, 137.9, 136.5, 135.8, 135.2, 127.3, 126.0, 107.1, 61.7, 61.6, 56.4, 32.8, 29.9, 15.6. The X-ray analysis of **4b** will be published elsewhere.

10,11,12-Trimethoxy-7,8-dihydro-4*H*-benzo[1,2]heptaleno-[5,6-*cd*]isoxazol-4-one, 3. The oxime **4a** (25 mg, 0.065 mmol) was dissolved in 92% methanol (18 ml). The mixture was heated until a clear solution was obtained. Sodium carbonate (28 mg) was added and the solution was refluxed for 3 h. After evaporation of the methanol, 2 M HCl was added and the aqueous solution was extracted twice with chloroform. The combined organic phases were dried and concentrated to a yellow solid. Purification with flash chromatography (ethyl acetate–heptane) yielded a yellow solid (17 mg, 77%), which could not be recrystallized. ν/cm^{-1} (film): 2937, 1628, 1577, 1507, 1490, 1344, 1250, 1198, 1143 and 1116; NMR, see Table 2; MS: $C_{19}H_{17}NO_5$, calc. 339.1107; found 339.1087.

X-Ray crystallography

Crystals of **1** and **2** were grown from ethanol solutions. The crystals were pale yellow (001) and (100) plates, respectively. Crystallographic data were collected on a modified Nicolet P3 diffractometer under control of local software.[25] Graphite-monochromated Cu-Kα radiation ($\lambda = 1.5418$ Å) was used. Table 6 summarizes the crystal data, data collection and structure refinement. Three intensity standards were checked every 60 min. They showed only negligible decay. The intensity data

Table 6 Crystallographic data for **1** and **2**

	1	**2**
Formula	$C_{20}H_{20}O_5S$	$C_{20}H_{19}NO_5S$
M	372.43	385.43
Crystal system	Triclinic	Triclinic
Space group	$P\bar{1}$	$P\bar{1}$
a/Å	7.209(2)	7.791(2)
b/Å	11.027(3)	10.706(2)
c/Å	12.940(3)	11.110(2)
α/°	109.17(3)	85.13(2)
β/°	102.66(3)	78.70(4)
γ/°	99.80(5)	75.77(2)
V/Å^3	914.8(7)	880.2(4)
Z	2	2
F(000)	392	404
D_c/g cm^{-3}	1.352	1.454
Crystal size/mm	0.30 × 0.25 × 0.07	0.25 × 0.19 × 0.08
μ/mm^{-1}	1.82	1.93
Transmission	0.870–1.0	0.923–1.0
Decay (%)	−0.42	−0.01
Data measured	5420	5244
No. unique	2710	2622
R_{int}	0.076	0.069
$I > 3\sigma(I)$	1850	1670
No. parameters	316	308
R	0.054	0.066
R_w	0.059	0.095
Weights, w^{-1}	$\sigma^2(F) + 0.00032F^2$	$\sigma^2(F) + 0.0009F^2$
Residual peaks/e Å^{-3}	0.32/−0.32	0.35/−0.49

were corrected for Lorentz, polarization and absorption effects (ψ scans). The structures were solved with direct methods and difference electron density calculations using the TEXSAN program package.[26] The full-matrix least-squares refinements minimized the function $\Sigma w(|F_o| - |F_c|)^2$. In the final refinements all non-hydrogen atoms were assigned anisotropic thermal parameters. Most of the hydrogen atoms could be located in the maps and were refined with isotropic temperature factors. The three hydrogen atoms of the methylthio group of **2** were introduced at the calculated positions and kept fixed during the refinements. Neutral atomic scattering factors and anomalous dispersion corrections were taken from *International Tables for X-Ray Crystallography*.[27] Atomic coordinates, bond lengths and angles, and thermal parameters, for **1** and **2** have been deposited at the Cambridge Crystallographic Data Centre (CCDC). For details of the deposition scheme, see 'Instructions for Authors', *J. Chem. Soc., Perkin Trans. 2*, 1997, Issue 1. Any request to the CCDC for this material should quote the full literature citation and the reference number 188/75.

Spectroscopy

^{1}H and ^{13}C NMR spectra were recorded on Varian XL-300 or Bruker DRX 400 spectrometers, using the solvent peak, usually $CDCl_3$, as internal shift standard. The dynamic NMR experiment is described by Sandström.[28] Temperature calibration of the NMR spectrometer was performed with methanol according to the method described by van Geet.[29] The rate constants were evaluated by visual fitting of the experimental spectra to spectra calculated by the McConnel classical formalism,[30] or by DNMR5.[31] The evaluations of T_2 and $\delta\nu$ values for bandshape calculations were performed as previously described.[32] The enthalpies and entropies of activation were obtained by a linear regression analysis of ln (k/T) *versus* ($1/T$) according to the Eyring equation.[33] Errors in activation parameters have been given with the assumption that the temperature could be determined with an accuracy of ±0.5 K.[34]

Fluorescence experiments were recorded on a Turner Designs TD-700 Fluorometer equipped with 300–400 nm excitation and 410–600 emission filters. Standard 13 mm round glass test-tube cuvettes were used. The total volume of the solutions was 3 ml. Kinetic and displacement experiments were carried out at 21 ± 1 °C. Tubulin from microtubule protein from bovine brain, used in the fluorescence experiments, was obtained from Dr M. Wallin, Göteborg University. Experiments were performed in standard solutions containing 100 mM Pipes (1,4-piperazinediethanesulfonic acid) buffer, 0.5 mM $MgSO_4$ and 0.15 mM GTP at pH 6.8.

Molecular mechanics

MM analysis was performed using the MM2(91) force field[35] implemented in the MACMIMIC program package.[36]

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