SYNTHESIS AND TUBULIN INTERACTION OF THIOCOLCHICINES CONTAINING AN ISOTHIOCYANATO GROUP. SYNTHESIS OF C(2)-DEUTERATED AND C(2)-^{14}C-LABELED 7-ISOTHIOCYANATODEACETAMIDOTHIOCOLCHICINE

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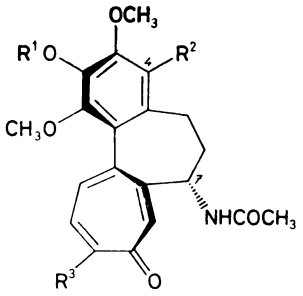
This paper is dedicated to Dr Miroslav Protiva, a friend of long acquaintance, on the occasion of his 70th birthday.

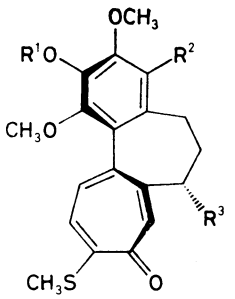
Synthesis of isothiocyanato substituted thiocolchicines *XI*—*XIV* is described. Introduction of an isotope label is demonstrated with the deuterated isothiocyanate *XII* and the ^{14}C-labeled analog *XIII*. These isothiocyanates inhibit tubulin polymerization at low concentration. In addition, the ^{14}C-labeled *XIII* forms covalent bond(s) with tubulin. Unfortunately, the covalent reaction while rapid, is not inhibited by preincubation of tubulin with colchicine. The covalent interaction of *XIII* with tubulin thus appears to be nonspecific, limiting its use as a marker of the colchicine binding site on tubulin.

Tubulin, a 100,000 dal protein and a major component of microtubules, is composed of alpha and beta subunits. Colchicine (*I*), the best known of the spindle toxins, binds non-covalently to each tubulin dimer in a 1 : 1 stoichiometry, forming a colchicine tubulin complex which dissociates slowly and which inhibits microtubule assembly[1]. It is presently not known whether the colchicine-binding site on tubulin (CTBS) is located on the alpha or at the beta subunit, or perhaps bridges the two subunits. An understanding of this process at a molecular level would be greatly facilitated by the isolation and characterization of the peptide core of CTBS. Elucidating CTBS would be useful in designing "improved" spindle toxins, of potential medical use in a variety of disorders, such as gout, Familial Mediterranean Fever (FMF), cirrhosis of the liver, phelebitis and neoplastic diseases[2,3]. It is speculated that covalently bound marker molecules of CTBS could be obtained by introducing into the colchicine molecule chemically reactive groups at noncritical positions. This should permit characterization of CTBS possible by physical methods, X-ray

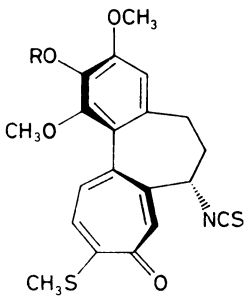
analysis, or through amino acid sequencing[4]. Attempts to prepare CTBS-marker molecules were made by introducing bromine or fluorine into the acetamidogroup[5,6], by preparing azides of C(9)- and C(10)-demethoxycolchicines[7] and by preparing photoactive azide analogs modified in the acetamido group[8]. These efforts afforded interesting compounds, but thus far they have not been exploited to provide much detailed information about CTBS, other than suggesting that the acetamido side chain of colchicine interacts primarily with the alpha subunit[5,7].



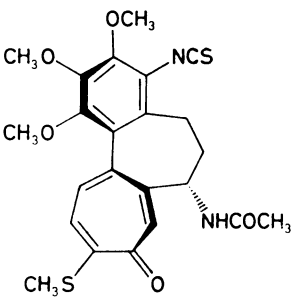
I, $R^1 = CH_3$; $R^2 = H$; $R^3 = OCH_3$
II, $R^1 = CH_3$; $R^2 = H$; $R^3 = SCH_3$
III, $R^1 = R^2 = H$ $R^3 = SCH_3$
IV, $R^1 = CD_3$; $R^2 = H$; $R^3 = SCH_3$
V, $R^1 = {}^{14}CH_3$; $R^2 = H$; $R^3 = SCH_3$
VI, $R^1 = CH_3$; $R^2 = CHO$; $R^3 = SCH_3$



VII, $R^1 = CH_3$; $R^2 = H$; $R^3 = NH_2$
VIII, $R^1 = CD_3$; $R^2 = H$; $R^3 = NH_2$
IX, $R^1 = {}^{14}CH_3$; $R^2 = H$; $R^3 = NH_2$
X, $R^1 = CH_3$; $R^2 = NH_2$; $R^3 = NHCOCH_3$



XI, R = CH_3
XII, R = CD_3
XIII, R = $^{14}CH_3$



XIV

To prepare potentially stable markers of CTBS we considered it worthwhile to introduce the isothiocyanato group (–NCS) into colchicine at noncritical positions. The NCS group was successfully introduced into morphinans[9] and other antinoci-

ceptive molecules[10] allowing marking and recognition of subpopulations of opiate receptor biopolymers[11]. The NCS-label was preferred over the NCO-label because of its greater stability in an aqueous milieu[12]. Noncritical positions in thiocolchicine (*II*) are the acetamido group at C(7) which can be omitted, or replaced with other acylamido groups[4], and C(4) which is available for electrophilic substitution, as shown with the potent 4-formylthiocolchicine[13] (*VI*). In planning our project we decided to introduce the NCS-labels in the highly potent thiocolchicine[14] (*II*), rather than in colchicine (*I*). Thiocolchicine (*II*) is not sensitive to acid and affords with Lewis acid the 2-demethyl analog *III*, which can be used to introduce isotope labels at this position[15]. Although the methoxy group at C(2) is metabolically unstable[16] its chemical stability should permit analysis of radiolabeled CTBS complexes. We report here the synthesis of isothiocyanates *XI* and *XIV* with the isothiocyanato group in position C(7) and C(4) respectively, and the synthesis of the deuterated analog *XII* and analog *XIII* with a ^{14}C-label at the C(2)–methoxy group.

RESULTS AND DISCUSSION

Chemistry

Amines *VII* and *X* were prepared by the published procedures[17,18] and amine *X* is additionally characterized. Conversion of *VII* and *X* into isothiocyanates *XI* and *XIV* was accomplished with thiophosgene in chloroform as published[10,11]. Alkylation of *III* with CD_3I was accomplished in acetone in the presence of presence of potassium carbonate and *V* was similarly obtained from *III* using [^{14}C]methyl iodide. The acetamido group in *IV* and *V* was hydrolysed with 20% sulfuric acid as described for the preparation[17] of *VII*. All the isothiocyanates are optically active and they are characterized by physical data including optical rotations. The ^{1}H NMR spectrum of all deuterated compounds showed the presence of only two methoxy groups as singlets. The singlet of the C(2)—OCH_3 protons observed at δ 3·93 in thiocolchicine disappeared in the ^{1}H NMR spectrum of the deuterated analogue *IV*. Reaction of isothiocyanate *XI* with ethylamine gave a crystalline thiourea.

Biological Evaluation

All compounds were evaluated for their effect on the polymerization of purified tubulin dependent on glutamate and GTP, as described previously[18]. In brief, 10 μM, tubulin (1·0 mg/ml) and varying concentration of the agent under study were preincubated for 15 min at 37°C before polymerization was initiated by the addition of 0·4 mM GTP. The resulting data was then used to determine graphically the drug concentration required to inhibit the reaction by 50%. At least three independent determinations were made with each compound. Table I summarizes the data

obtained in two series of experiments, in both of which thiocolchicine was used as the standard for comparison. In our earlier work[18] we found that thiocolchicine was 1·5 to 2 times as potent as colchicine as an inhibitor of tubulin polymerization.

Experiment A shown in Table I compares the activities of 2-demethylthiocolchicine (*III*), deacetylthiocolchicine (*VII*), and 7-isothiocyanato-deacetamidothiocolchicine (*XI*) to those of thiocolchicine (*II*) and colchicine (*I*). Each of these modifications in thiocolchicine resulted in small losses of activity relative to the parent compound, but the three agents were nonetheless quite active as inhibitors of tubulin polymerization, since they were at least as active as colchicine itself.

Experiment B in Table I presents data with compounds derivatized at position C(4) of thiocolchicine. The first compound prepared, 4-formylthiocolchicine (*VI*), was as active as thiocolchicine. Substitution with an amino group (compound *X*) or an isothiocyanato group (compound *XIV*) resulted in some loss of potencial. Despite their reduced activity relative to thiocolchicine (*II*), these latter two agents also are of the same order of potency as colchicine and represent compounds with significant antitubulin activity.

The significant activity of compound *XI* as an inhibitor of tubulin polymerization led us to synthesize a ^{14}C-labeled version of the agent, compound *XIII*. The initial studies with this material were promising in that it interacted with tubulin, and a significant amount of radiolabel remained bound to the protein following its denaturation in 8M urea. Since radiolabeled colchicine is completely released from tubulin in following urea treatment[19], this indicated covalent bond formation

TABLE I

Inhibition[a] of tubulin polymerization by thiocolchicine derivatives

Compound added	IC_{50}, μmol/l (*S.D.*)	
	Experiment A	Experiment B
Colchicine (*I*)	2·4 (0·08)[b]	
Thiocolchicine (*III*)	1·3 (0·04)[b]	1·6 (0·2)
2-Demethylthiocolchicine (*III*)	2·0 (0·2)[b]	
4-Formylthiocolchicine (*VI*)		1·5 (0·2)
Deacetylthiocolchicine (*VII*)	2·0 (0·2)[b]	
4-Aminothiocolchicine (*X*)		2·4 (0·1)
7-Isothiocyanato-deacetamidothiocolchicine (*XI*)	2·3 (0·2)	
4-Isothiocyanato-thiocolchicine (*XIV*)		2·6 (0·1)

[a] The experimental procedure was described in detail previously[18]. Data are presented in terms of the concentration of each compound required to inhibit the extent of tubulin polymerization at 20 min by 50% (IC_{50} value). [b] These data were presented previously[18].

between the protein and *XIII*. Subsequent work, however, has not been promising. Radiolabel was incorporated into both the alpha and beta subunits. Even more discouraging, the covalent reaction was not temperature dependent, unlike the binding of colchicine and thiocolchicine to tubulin[14]. Finally, no specificity could be documented for the covalent reaction, since it was not reduced if tubulin were pre-incubated with colchicine, presumably saturating the CTBS.

At this point we do not know if the lack of specificity of compound *XIII* is due to excessive reactivity of the isothiocyanato group or to its particular position in the thiocolchicine molecule. We plan to prepare a radiolabeled version of compound *XIV*, both to answer this question and as a second attempt to develop a satisfactory chemical affinity analog of colchicine.

EXPERIMENTAL

Melting points (uncorrected) were determined with a Fisher–John apparatus. Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter in chloroform with the concentration specified at the temperature range 22—25°C. The UV spectra (λ_{max}, $CHCl_3$) were measured on a Hewlett–Packard 8450A UV/VIS spectrophotometer. IR spectra were recorded in $CHCl_3$ on a Beckman IR 4230 spectrometer (wavenumbers in cm^{-1}). ^1H NMR spectra were taken in $CDCl_3$ using a JEOL JNX-FX 300 spectrometer with tetramethylsilane as the internal reference. Chemical shifts are given in ppm (δ-scale), coupling constants (J) in Hz. Electron-impact mass spectra were obtained with a V. G. Micromass 7070F mass spectrometer (70 eV, source temperature 210°C). Thin layer chromatography plates (silica gel) were purchased from Analtech, Inc., Newark, DE, and silica gel Merck 60 (230—400 mesh) from Aldrich was used for column chromatography. The solvent system used for TLC analysis was $CHCl_3/CH_3OH$ (9 : 1) and $CHCl_3/CH_3OH/NH_4OH$ (9 : 0·9 : 0·1).

7-Isothiocyanato-deacetamidothiocolchicine (*XI*)

Deacetylthiocolchicine (50 mg, 0·13 mmol) was dissolved in $CHCl_3$ (10 ml) and saturated $NaHCO_3$ solution (10 ml) added and the mixture was stirred vigorously for 5—10 min under nitrogen. To this solution was added thiophosgene (0·01 ml) with ice-bath cooling and the reaction mixture was kept stirring for 30 min. The chloroform layer was separated, washed with a saturated $NaHCO_3$ solution and brine, dried (Na_2SO_4) and concentrated. The extract was passed through a small silica gel column using $CHCl_3$/ether as eluant. Crystallization from methanol/ligroin (80 : 20) yielded yellow crystals (30 mg, 60%): m.p. 148—150°C; $[\alpha]_D$ −344° (*c* 0·23, $CHCl_3$). IR spectrum: 2 050 (N=C=S). ^1H NMR spectrum: 2·44 s, 3 H (SCH_3); 3·69 s, 3 H (OCH_3); 3·90 s, 3 H (OCH_3); 3·91 s, 3 H (OCH_3); 4·67 m, 1 H (H-7); 6·54 s, 1 H (H-4); 7·03 d, 1 H (J = 10·49, Ar-H); 7·18 d, 1 H (J = 10·49, Ar-H); 7·47 s, 1 H (H-8). MS, *m/z*: 415 (M^+, 100%).

Reaction of *XI* with ethylamine: afforded thiourea derivative m.p. 231—232°C ($CHCl_3$/ether): $[\alpha]_D$ −146° (*c* 0·1, $CHCl_3$). MS, *m/z*: 460 (M^+).

2-Trideuteromethylthiocolchicine (*IV*)

2-Demethylthiocolchicine (*III*, 50 mg, 0·12 mmol) was dissolved in acetone (5 ml). To this solution was added anhydrous K_2CO_3 (173 mg, 1·2 mmol) and CD_3I (0·8 ml, 1·2 mmol).

Reaction mixture was stirred at 60°C for 1 h and then passed through celite, concentrated and crystallized from ethyl acetate/ether to give 2-trideuteromethylthiocolchicine (*IV*) as yellow crystal: (37 mg, 74%), m.p. 189°C; $[\alpha]_D$ −364° (*c* 0·15, $CHCl_3$). UV spectrum: 257, 292. 391 nm. IR spectrum: 1 670 (C=O, amide). ^{1}H NMR spectrum: 2·02 s, 3 H ($COCH_3$); 2·47 s, 3 H (SCH_3); 3·65 s, 3 H (OCH_3); 3·90 s, 3 H (OCH_3); 4·69 m, 1 H (H-7); 6·54 s, 1 H (Ar-H); 7·16 m, 1 H (NH); 7·20 d, 1 H (*J* = 10·5, Ar-H); 7·43 d, 1 H (*J* = 10·5, Ar-H); 7·63 s, 1 H (Ar-H). MS, *m/z*: 4·18 (M^+, 100%).

2-Trideuteromethyldeacetylthiocolchicine (*VIII*)

A solution of 2-trideuteromethylthiocolchicine (*IV*, 90 mg, 0·21 mmol) in 20% H_2SO_4 (10 ml) was stirred at 90°C for 6 h. Reaction mixture was poured on ice and saturated solution of $NaHCO_3$ was added to make the pH 7. The aqueous layer washed with brine, dried (Na_2SO_4), and concentrated. The crude extract was flash chromatographed on silica gel using $CHCl_3$/methanol (98 : 2) as eluant to give 2-trideuteromethyldeacetylthiocolchicine (*VIII*) as pure compound which was triturated with ether (41 mg, 52%): m.p. 196—197°C, $[\alpha]_D$ −345° (*c* 0·3, $CHCl_3$). ^{1}H NMR spectrum: 2·44 s, 3 H (SCH_3); 3·71 s, 3 H (OCH_3); 3·92 s, 3 H (OCH_3); 4·23 m, 1 H (H-7); 6·55 s, 1 H (H-4); 7·05 d, 1 H (*J* = 10·7, Ar-H); 7·28 d, 1 H (*J* = 10·7, Ar-H). CIMS, *m/z*: 337 ($M^+ + 1$).

2-Trideuteromethyl-7-isothiocyanato-deacetamidothiocolchicine (*XII*)

Same procedure as described for *XI*. Crystallization from methanol/ligroin afforded *XII* as yellow crystals (32 mg, 0·076 mmol, 59%): m.p. 142—144°C; $[\alpha]_D$ −622° (*c* 0·2, $CHCl_3$); UV spectrum: 255, 290 (sh), 389 nm. IR spectrum: 2 045 (N=C=S). ^{1}H NMR spectrum: 2·45 s, 3 H (SCH_3); 3·69 s, 3 H (OCH_3); 3·91 s, 3 H (OCH_3); 4·69 m, 1 H (H-7); 6·54 s, 1 H (H-4), 7·06 d, 1 H (*J* = 10·5, Ar-H); 7·21 d, 1 H (*J* = 10·5, Ar-H); 7·55 s, 1 H (H-8). MS, *m/z*: 4·18 (M^+, 100%).

2-[^{14}C]Methyl-7-isothiocyanato-deacetamidothiocolchicine (*XIII*)

Compound *III* was methylated with [^{14}C]methyliodide (5 mg, 2 000 mCi, 56·6 mCi/mmol) by the procedure described for *IV* to afford ^{14}C-labeled *V* which after deacetylation to *IX* followed by reaction with thiophosgene (as described for *XI* afforded 2-[^{14}C]methyl-7-isothiocyanato-deacetamidothiocolchicine (*XIII*, 2 mg, 5·6 mCi/mmol). All the ^{14}C-labeled compounds were characterized by TLC comparison with the non-labeled analogs.

4-Aminothiocolchicine (*X*)

4-Aminothiocolchicine (*X*) was prepared from thiocolchicine by the procedure described[13] and isolated and fully characterized: m.p. 116°C (ethyl acetate/ether); $[\alpha]_D$ −190° (*c* 0·14, $CHCl_3$). UV spectrum: 245, 368 nm. IR spectrum: 3 300 (NH); 1 725 (C=O, tropolone ring); 1 670 (C=O, amide). ^{1}H NMR spectrum: 1·93 s, 3 H ($COCH_3$); 2·35 s, 3 H (SCH_3); 3·48 s, 3 H (OCH_3); 3·86 s, 3 H (OCH_3); 3·90 s, 3 H (OCH_3); 4·56 m, 1 H (H-7); 6·97 d, 1 H (*J* = 10·4, H-10); 7·21 d, 1 H (*J* = 10·4, H-11). MS, *m/z*: 431 ($M^+ + 1$).

4-Isothiocyanato-thiocolchicine (*XIV*)

4-Aminothiocolchicine (*X*, 30 mg, 0·06 mmol) was converted into 4-isothiocyanato-thiocolchicine (25 mg), by the same procedure as described for *XI* and crystallized from ethyl acetate/ether:

m.p. 142–143°C; $[\alpha]_D$ −142° (*c* 0·33, $CHCl_3$). UV spectrum: 257, 367 nm. IR spectrum: 2 005 (N=C=S). ^{1}H NMR spectrum: 1·93 s, 3 H ($COCH_3$); 2·36 s, 3 H (SCH_3); 3·56 s, 3 H (OCH_3); 3·90 s, 3 H (OCH_3); 3·96 s, 3 H (OCH_3); 4·48 m, 1 H (H-7); 6·87 bs, 1 H (NH); 3·97 d, 1 H (*J* = 10·4, H-10); 7·15 d, 1 H (*J* = 10·4, H-11); 7·28 s, 1 H (H-8). MS, *m/z*: 472 (M^+, 100%).

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