SYNTHESIS OF ^{14}C LABELLED ELECTROPHILIC LIGANDS OF THE COLCHICINE BINDING SITE OF TUBULIN: CHLOROACETATES OF DEMETHYLTHIOCOLCHICINES AND OF N-ACETYLCOLCHINOL; ISOTHIOCYANATE OF 9-DEOXY-N-ACETYLCOLCHINOL.

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SUMMARY

^{14}C-Chloroacetates of 2-demethylthiocolchicine **7** (specific activity 55.0 mCi/mmol, radiochemical yield 26.1%), and of 3-demethylthiocolchicine **8** (specific activity 55.0 mCi/mmol, radiochemical yield 5.7%) were synthesized and found to covalently bind with high specificity to the β-subunit of tubulin. The ^{14}C-chloroacetate of *N*-acetylcolchinol **5** (specific activity 56.0 mCi/mmol, radiochemical yield 7.8%) and the ^{14}C-isothiocyanate **6** (specific activity 50.0 mCi/mmol, radiochemical yield 32%) were also prepared and found to react covalently with tubulin but in a nonspecific manner. With the radiolabelled chloroacetates **7** and **8** two compounds are now available to further characterize the colchicine binding site on the β subunit of tubulin.

Key words: Colchicine, Affinity Ligand, Tubulin, Microtubule, ^{14}C-Radiolabelled Ligand

INTRODUCTION

Microtubules are found in all eukaryotic cells and are essential components for the maintenance of cellular morphology and for cell division. Their formation and disassembly, utilizing free tubulin as a building block, is a highly regulated process that is not well understood at the cellular level.[1] For this reason the study of the interaction with tubulin of the potent drugs known as spindle toxins (colchicine, podophyllotoxin, vincristine, vinblastine, taxol etc...) is of major importance and the subject of intensive research.[2,3] The localization of the colchicine binding site of tubulin has been attempted in several laboratories notably through photolabelling of the protein.[4-7] Preferential labelling of both the α-subunit[4,5] and β-subunit[6,7] of tubulin has been reported, leading to a controversy about the exact location of the

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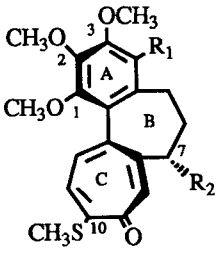
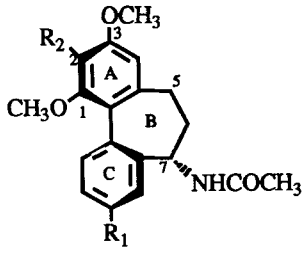
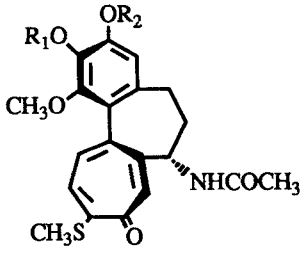
colchicine binding site. Except for direct photoaffinity reaction of colchicine itself with tubulin,[7] reactive moieties have been introduced in the B-ring side chain at position C(7).



1 $R_1 = H$, $R_2 = NHCOCH_3$
3 $R_1 = NCS$, $R_2 = NHCOCH_3$
4 $R_1 = H$, $R_2 = NCS$



2 $R_1 = R_2 = OCH_3$
5 $R_1 = O^{14}COCH_2Cl$, $R_2 = OCH_3$
6 $R_1 = NCS$, $R_2 = O^{14}CH_3$



7 $R_1 = {}^{14}COCH_2Cl$, $R_2 = CH_3$
8 $R_1 = CH_3$, $R_2 = {}^{14}COCH_2Cl$

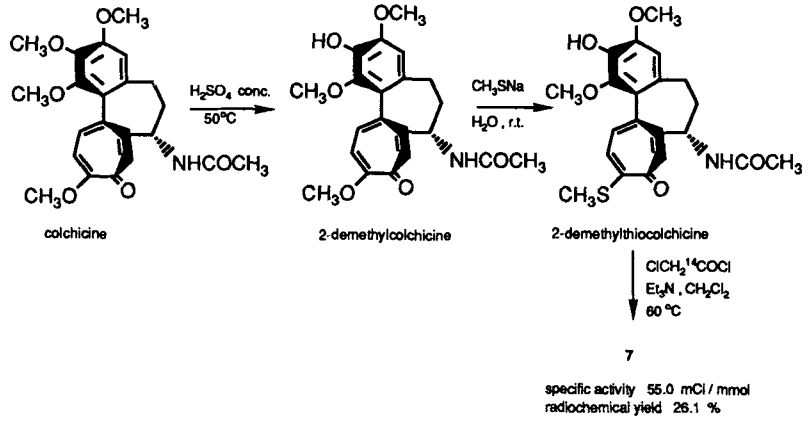
Thiocolchicine **1** and *N*-acetylcolchinyl methyl ether **2** bind to tubulin with high affinity[8], and represent useful templates to design ligands suitable for a covalent interaction with the colchicine binding site on tubulin. We now report the synthesis of ^{14}C-labelled chloroacetoxy derivatives of thiocolchicine, **7** and **8**, which were found to react with high specificity with the tubulin β-subunit. Radiolabelled 9-chloroacetoxy-*N*-acetylcolchinol **5** was also synthesized in an attempt to expand the mapping of the receptor. Finally, we describe the synthesis of the radiolabelled isothiocyanate **6**, previously obtained in a non-radiolabelled form[8], and found to interact very efficiently with tubulin. The isothiocyanate functionality was originally chosen as a electrophilic center for its high stability in aqueous solvent and the excellent results obtained in covalent marking and identification of opiate receptors[10,11]. The isothiocyanates **3** and **4**, with the isothiocyanato group on ring A and B respectively, were prepared earlier[12] but were found to interact nonspecifically with tubulin.

SYNTHESIS

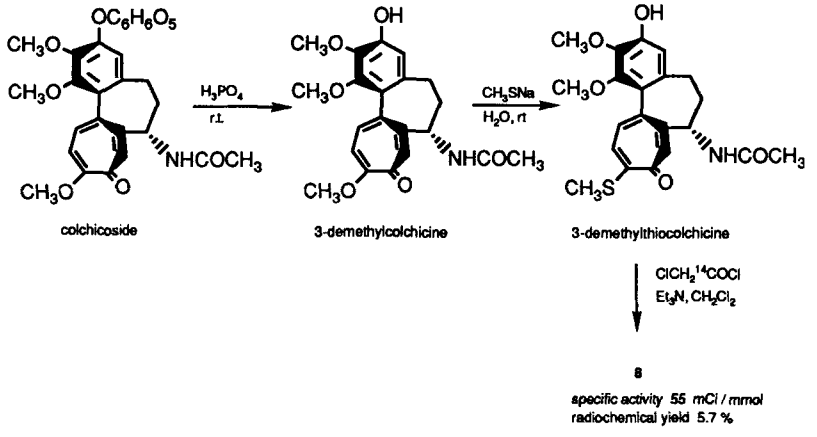
Synthesis of 2- and 3-demethylthiocolchicine was accomplished from colchicine and colchicoside (Schemes 1 and 2)[13,14]. Reaction of the 2- and 3-demethylthiocolchicine with ^{14}C-chloroacetyl chloride in dichloromethane

solution in the presence of triethylamine gave chloroacetate derivatives **7** and **8**, respectively. The yield of the reaction was surprisingly much higher for **7** where the acetoxylation occurred in a sterically more hindered position as compared to **8**.

Scheme 1
Synthesis of 2-chloroacetoxy-2-demethylthiocolchicine.



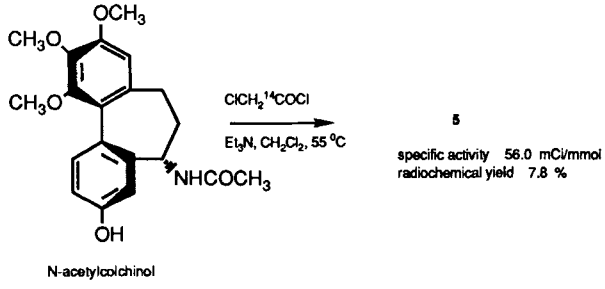
Scheme 2
Synthesis of 3-chloroacetoxy-3-demethylthiocolchicine.



Synthesis of radiolabelled 9-chloroacetoxy-*N*-acetylcolchinol **5** (Scheme 3) followed the same procedure starting from *N*-acetylcolchinol obtained from colchicine[15].

Scheme 3

Synthesis of 9-chloroacetoxy-*N*-acetyl-colchinol.



Introduction of the electrophilic isothiocyanato group at C(9) of the allocolchicine derivative **6** was made following a 7-step procedure elaborated earlier for the preparation of the cold material[9]. The radiolabelled compound was obtained by methylation of the hydroxyl group in position C(2) with ^{14}C-methyl iodide. The radioactive incorporation was efficient giving a final radiochemical yield of 32%.

DISCUSSION

^{14}C-Chloroacetoxythiocolchicine derivatives **7** and **8** reacted covalently with the colchicine binding site on tubulin with a β-subunit:α-subunit marking ratio of about 4:1.[17] The specificity of the marking was clearly demonstrated by a decrease in the incorporation of the radiolabelled ligand of more than 80% after preincubation of tubulin with podophyllotoxin, a potent competitive inhibitor of colchicine.[17] Compounds **7** and **8** are the first electrophilic colchicine analogs to react covalently with tubulin with such a high specificity. The digestion of tubulin after covalent reaction with these radiolabelled markers is now underway. This should allow a sequencing of the β-subunit peptides constituting the colchicine binding site.

The experimental results with radiolabelled electrophilic markers **5** and **6**, functionalized in position C(9), were disappointing in the lack of specificity in their covalent reaction with the protein.

EXPERIMENTAL

GENERAL

Melting points were determined on a Thomas Hoover capillary apparatus and are uncorrected. Chemical ionization mass spectra (CIMS) were obtained using a Finnigan 1015 mass spectrometer. ^{1}H-NMR spectra were taken using a Varian XL-300 spectrometer. Fourier transformed infrared spectra (FT-IR) were obtained from $CHCl_3$ solutions of the compounds using a Bio-Rad FTS-45 IR spectrometer. Specific rotation ($[\alpha]_D$) values were

determined at the sodium D-line (589 nm) at 25 °C using a Perkin-Elmer 241-MC polarimeter. Analytical and preparative thin layer chromatography (TLC) were performed on Analtech 250 μm GHLF and 2000 μm GF silica gel plates, respectively.

3-Chloroacetoxy-3-demethylthiocolchicine (8) and 2-chloroacetoxy-2-demethylthiocolchicine (7).

Preparation of the non-radiolabelled material: 3-demethylthiocolchicine (220 mg, 0.57 mmol), was dissolved in CH_2Cl_2 (20 mL). Triethylamine (100 μL, 0.71 mmol) was added and the mixture was cooled in ice before addition of chloroacetyl chloride (50 μL, 0.63 mmol). The solution was stirred 12 h at 60 °C. After cooling, the dichloromethane solution was washed with H_2O and dried (Na_2SO_4). Evaporation of the solvent gave a residue which was purified by chromatography (SiO_2, CH_2Cl_2/MeOH 95:5).

Compound **8** was crystallized from $(i\text{-Pr})_2O$ as yellow crystals (106 mg, 48%): mp 137-138 °C; $[\alpha]_D$ -152 ° (c=0.16, MeOH); FT-IR ($CHCl_3$) 3443, 3006, 2942, 2921, 1782, 1765, 1679, 1609, 1551, 1479, 1308, 1225, 1138, 1067, 1010 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.65-1.78 (m, 1H, CH_2), 1.91 (s, 3H, $COCH_3$), 2.05-2.2 (m, 1H, CH_2), 2.22-2.33 (m, 1H, CH_2), 2.35 (s, 3H, CH_3S), 2.42-2.48 (m, 1H, CH_2), 3.56 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 4.26 (s, 1H, $COCH_2Cl$), 4.49-4.55 (m, 1H, 7-H), 6.21 (br s, 1H, NH), 6.62 (s, 1H, Ar-H), 6.96 (d, J=10.5 Hz, 1H, Ar-H), 7.13-7.19 (m, 2H, Ar-H); CIMS (NH_3) 478 (MH^+), 402.

Compound **7** was obtained from 2-demethylthiocolchicine (220 mg, 0.57 mmol), as a yellow powder which was recrystallized from acetone/$(i\text{-Pr})_2O$ as bright yellow crystals (180 mg, 66%): mp 125 °C; $[\alpha]_D$ -182 ° (c=0.12, MeOH); FT-IR ($CHCl_3$) 3291, 2934, 2855, 1780, 1709, 1673, 1660, 1605, 1546, 1487, 1322, 1223; ^{1}H-NMR ($CDCl_3$) δ 1.8-1.9 (m, 1H, CH_2), 2.0 (s, 3H, $COCH_3$), 2.2-2.35 (m, 1H, CH_2), 2.43 (s, 3H, CH_3S), 2.45-2.65 (m, 2H, CH_2), 3.58 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 4.39 (s, 2H, $ClCH_2CO$), 4.60-4.70 (m, 1H, 7-H), 6.21 (m, 1H, NH), 6.61 (s, 1H, Ar-H), 7.02 (d, J=10.5 Hz, 1H, Ar-H), 7.22 (s, 1H, Ar-H), 7.29 (d, 1H, Ar-H); CIMS (NH_3) m/z 478 (MH^+), 402.

Preparation of the radiolabelled material:

Compound **8:** 3-demethylthiocolchicine (11 mg, 0.028 mmol) was dissolved in CH_2Cl_2 (3 mL). Et_3N (40 μL, 18 eq.) was added and the mixture was cooled (ice-H_2O bath) to 5 °C prior to addition of chloroacetyl chloride (55.4 mCi/mmol, 1 mCi, 0.018 mmol) diluted in CH_2Cl_2 (4 mL). The solution was heated in a sealed flask for 48 h at 55 °C. After cooling, the mixture was concentrated by applying a stream of nitrogen. The residue was separated by preparative TLC (SiO_2, CH_2Cl_2/MeOH 95/5) to afford the radiolabelled material **8** (0.5 mg) which was dissolved in DMSO to a final concentration of 10^{-2} M (specific activity 55.0 mCi/mmol, radiochemical yield 5.7 %).

Compound **7**: 2-demethylthiocolchicine (7 mg, 0.018 mmol) was reacted in the same manner to afford radiolabelled compound **2** (2.5 mg, specific activity 55.0 mCi/mmol, radiochemical yield 26.1 %).

9-Chloroacetoxy-*N*-acetyl-colchinol (5).

Preparation of the non-radiolabelled material: *N*-Acetylcolchinol (50 mg, 0.14 mmol) was dissolved in CH_2Cl_2 (10 mL). Triethylamine (0.2 mL, 10 eq.) was added and the solution was cooled on ice before addition of chloroacetyl chloride (14 μL, 0.18 mmol). The mixture was stirred under N_2 at 50 °C for

24 h. The organic solution was washed with H_2O and dried (Na_2SO_4). Filtration and evaporation of the solvent gave a residue which was purified by preparative TLC (SiO_2). Compound **5** was obtained as an off-white powder (25 mg, 41%) and a 4/1 mixture of atropisomers as determined by ^{1}H-NMR: mp 78-79 °C; $[\alpha]_D$ -87 ° (c=0.16, MeOH); FT-IR (neat) 3291.7, 2937.8, 2854.2, 1770.4, 1739.1, 1650.4, 1556.9, 1541.2, 1482.4, 1457.0, 1403.4, 1322.6, 1238.0, 1149.0, 1102.2 cm^{-1}; ^{1}H-NMR ($CDCl_3$), *major isomer*, δ 2.07 (s, 3H, $COCH_3$), 2.24-2.56 (m, 4H, CH_2), 3.52 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 4.34 (s, 2H, CH_2Cl), 4.74-4.83 (m, 1H 80%, 7-H), 5.74-5.88 (m, 1H , N-H), 6.58 (s, 1H, 4-H), 7.08 (s, 1H, 8-H), 7.09 (d, J=7.1 Hz, 10-H), 7.17 (d, J=7.3 Hz, 10-H), 7.52 (d, J=8.8 Hz, 11-H); ^{1}H-NMR ($CDCl_3$), *minor isomer*, δ 2.07 (s, 3H, $COCH_3$), 2.24-2.56 (m, 4H, CH_2), 3.52 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 4.34 (s, 2H, CH_2Cl), 5.08-5.16 (m, 1H, 7-H), 5.24-5.32 (m, 1H, N-H), 6.58 (s, 1H, 4-H), 7.08 (s, 1H, 8-H), 7.09 (d, J=7.1 Hz, 10-H), 7.17 (d, J=7.3 Hz, 10-H), 7.52 (d, J=8.8 Hz, 11-H); EIMS m/z 433 (M^+), 391, 374.

Preparation of the radiolabelled material: *N*-acetylcolchinol (14 mg, 0.031 mmol) was reacted in the same manner as for the preparation of radiolabelled compounds **7** and **8** to afford radiolabelled chloroacetate **5** (0.6 mg, specific activity 56.0 mCi/mmol, radiochemical yield 7.8 %).

9-Isocyanato-9-deoxy-*N*-acetylcolchinol (6).

Preparation of the radiolabelled material: Isothiocyanate **6** was prepared by published procedure[8], using radiolabelled $^{14}CH_3I$ (50 mCi/mmol, 2 mCi, 0.04 mmol). Isothiocyanate **6** was obtained as a white powder (specific activity 50 mCi/mmol, radiochemical yield 32 %).

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