N-DIDEUTEROBUTYRYLDEACETYLCOLCHICINE: PROBES TO STUDY COLCHICINE BINDING SITES ON TUBULIN PROTEIN

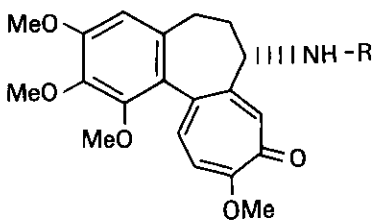
Maria A. Iorio and Antonio Doldo
Laboratory of Pharmaceutical Chemistry, Istituto Superiore di Sanita, 00161 Rome, Italy
Arnold Brossi
Section on Medicinal Chemistry, Laboratory of Chemistry, NIADDK, National Institute of Health, Bethesda, Maryland 20205, U.S.A.
Colin F. Chignell
Laboratory of Molecular Biophysics, National Institute of Environmental Health Services, Research Triangle Park, North Carolina 27709, U.S.A.

Abstract——N-Acryloyl- and N-crotonyldeacetylcolchicine prepared from deacetylcolchiceine by conventional methods showed good binding to tubulin protein in vitro. N-Dideutero-butyryldeacetylcolchicine, prepared from deacetylcolchicine with dideuterobutyryl chloride behaved similarly, suggesting that tritiated analogs of N-propionyl- and N-butyryl-deacetylcolchicine should be useful probes to study the colchicine binding site on tubulin protein.

Colchicine (1), a well known antimitotic agent, biosynthetically derived from androcymbine, binds exceptionally well to tubulin protein, and it is believed that this process is responsible for many of its biological effects.[1,2] It is thought that marking and characterizing the colchicine binding site on tubulin, identical to that of podophyllotoxine,[3] would greatly help in the understanding on how colchicine produces its biological effects and could possibly be useful in designing novel antitumor agents based on colchicine's structure. Although colchinoids containing spin-labels[4,5] and UV-sensitive substituents[6] have been prepared, none of these labels so far has proven useful to accomplish the objective. The finding that N-propionyl- and N-butyryldeacetylcolchicine were equally potent as colchicine in the tubulin binding assay in vitro, showing in addition similar profiles as colchicine in the P388 lymphocytic leukemia assay and in acute toxicity assays,[7] suggested that analogs with a tritium label in the N-acyl side chain could represent another type of affinity label for studying the colchicine binding site on tubulin.

N-Crotonyl- (3) and N-acryloyldeacetylcolchicine (4) intended to be used for deuteration mimicking tritiation, were prepared from a crude mixture of deacetylcolchicine (2) and the corresponding

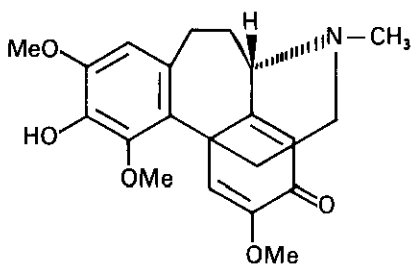
iso-form, obtained by the Raffauf procedure.[8] Acylation with crotonyl chloride and acryloyl chloride in dichloromethane in the presence of triethylamine[7] afforded 3 and 4 respectively, together with the corresponding iso-forms. Chromatography on silica gel with chloroform containing 5% methanol and 0.5% ammonia afforded, as usually, first the natural isomers 3 and 4, before the iso-isomers were eluted. Model experiments to prepare a deuterated analog of N-butyryldeacetyl-colchicine (6) with 2 deuterium atoms in the acyl side chain, treating 3 with deuterium gas in 95% EtOH in the presence of Pd/C catalyst, failed to afford after absorption of 1 mol deuterium a cleanly deuterated compound. The mass spectrum of the deuterated material showed, besides the expected molecular peak at m/z 429 (16.5%) other peaks 1-4 m.u. higher, indicating that reduction of the tropolonic ring had occurred. When the absorption of deuterium gas was complete, a cluster of peaks appeared between 434-438 m/z in the product obtained, corresponding to an addition of 3-4 moles of deuterium.[9] It seemed, therefore, more convenient to first deuterate crotonic acid and to prepare the dideuterated colchinoid 5 by traditional chemistry. Dideuterated crotonic acid obtained by deuteration of crotonic acid in 95% EtOH over Pd/C catalyst was chlorinated with thionylchloride and the chloride used for the amidation of 2, affording 5 from the original mixture of isomers after chromatography on silica gel as the faster moving compound ($MeOH:CHCl_3$ = 9:1).



1 R = $CO-CH_3$ 2 R = H

3 R = $CO-CH=CH-CH_3$ 4 R = $CO-CH=CH_2$

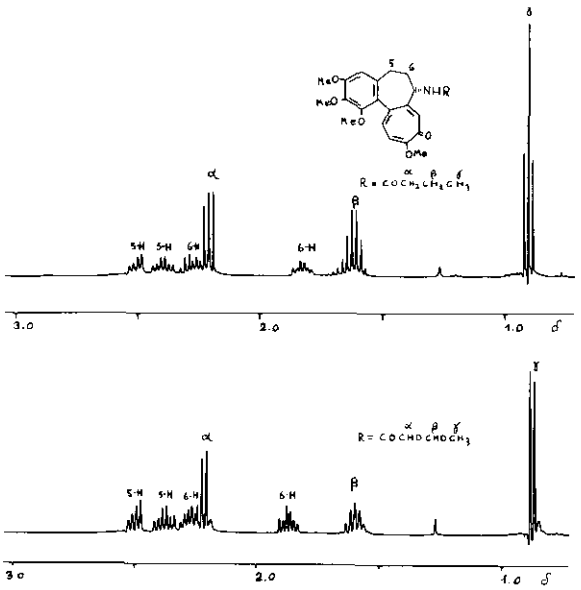
5 R = $CO-CHD-CHD-CH_3$ 6 R = $CO-CH_2-CH_2-CH_3$



Androcymbine

The product (5) obtained did not crystallize and resulted as an amorphous light-yellow powder (an identical material was obtained for 6). That compound 5 was deuterated only in the C-7 side-chain was confirmed by its ^{1}H-NMR spectrum run in parallel with the spectrum of 6 as shown in the Fig. In 5 the methyl protons (γ) appeared as a doublet δ 0.88; the methylene group attached to methyl (β) as a multiplet at δ 1.60, integrating for 1 H; and the methylene group near to carbonyl (α) as a doublet, at δ 2.21, integrating for 1 H.

In 6 the methyl signal (γ) was a triplet at δ 0.90; the methylene protons attached to methyl (β) a multiplet at δ 1.61, integrating for 2 H, and the methylene protons bound to carbonyl (α) a triplet δ 2.21, integrating for 2 H. Multiplicities in 5 are in complete agreement with this deuteration pattern in the N-acyl side chain.



The upfield region of the ^{1}H NMR spectra in $CDCl_3$ (Bruker AM 400 spectrometer) of N-butyryldeacetylcolchicine (6) and its deuterated analogue (5).

Colchicinoids with an unsaturated or a deuterated N-acyl side-chain of three to four carbon atoms were potent in a tubulin binding assay when compared to colchicine, and similarly toxic to mice, as shown in the Table.

Table : Binding of Unsaturated Colchicine Analogs to Rat Brain Tubulin Protein and Acute Toxicity in Mice.

COMPOUND	%inhib. of ^{3}H-colchicine binding[a]	LD_{50}[b]
3	67	3.4
4	82	17.4
5	79	NT
Colchicine	90	3.0

[a] Percentage by which the binding of ^{3}H-colchicine (2.5 μM) to tubulin is reduced in the presence of the colchicine analog (25 μM). [b]Toxicity found after a single intramuscular injection, in micromoles per kg.

EXPERIMENTAL

N-Crotonyldeacetylcolchicine (3).

To a cooled crude mixture of 2 and the corresponding iso-form prepared from 2 g of deacetylcolchiceine,[8] dissolved in 50 ml of dry dichloromethane and 1.5 ml of triethylamine, was added dropwise 1 ml of crotonoyl chloride dissolved in 5 ml of dichloromethane. After stirring at room temperature for 3 h (TLC monitoring; Al_2O_3, 2.5% MeOH-$CHCl_3$), the solution was poured into ice-water, adjusted to pH 6 with ammonia, and the dichloromethane layer isolated and dried over Na_2SO_4. After evaporation of solvent, the yellow residue was chromatographed on a column (2.5 x 80 cm) of silica gel-60 (0.04-0.06 mm) packed with chloroform and eluted with the same solvent containing 2.5% MeOH and 0.5% NH_4OH. N-Crotonyldeacetylcolchicine (3), eluted more readily than the iso-form; was crystallized from ethyl acetate to yield 310 mg; mp 219-221°C, $[\alpha]_D^{21}$ -127.0° (c, 1.17, $CHCl_3$). EIMS, m/z (rel. abundance): 425 (M^+, 69); 397 (M^+ - CO, 41); 356 (M^+ - COR, 19); 340 (M^+ - NH_2COR, 15); 328 (M^+ -(CO + COR), 21); 312 (M^+ - (CO + NH_2COR), 100); 69 ($COCH{=}CHCH_3$, 85) 41 ($CH{=}CHCH_3$, 98). Anal. Calcd. for $C_{24}H_{27}NO_6.1/2\ H_2O$: C, 66.36; H, 6.50; N, 3.22. Found: C, 66.30; H, 6.45, N, 3.25.

The second eluted component, N-crotonyldeacetylisocolchicine was obtained as an amorphous yellow powder and not further characterized.

N-Acryloyldeacetylcolchicine (4) was obtained in a similar manner and yield from a mixture of 2 and acryloyl chloride; mp 193-196°C from ethyl acetate, $[\alpha]_D^{20}$ = -122.5° (c, 0.65, $CHCl_3$); EIMS, m/z (rel. abundance): 411 (M^+, 52); 383 (M^+ - CO, 37); 356 (M^+ - COR, 10); 340 (M^+ - NH_2COR, 11) 328 (M^+ - (CO + COR), 13); 312 (M^+ -(CO + NH_2COR), 100); 55 ($COCH{=}CH_2$, 74). Anal. Calcd. $C_{23}H_{25}NO_6.1/2\ H_2O$: C, 64.33; H, 6.34; N, 3.26. Found: C, 64.72; H, 6.23, N, 3.12.

Dideuterobutyric acid

Crotonic acid (8 g, 0.1 mole) dissolved in 50 ml of 95% ethanol was deuterated at room temperature, under atmospheric pressure over 5% Pd/C (1 g) until the theoretical amount of deuterium was absorbed (ca. 2.2 l). The catalyst was filtered, the solvent evaporated under low vacuum and the liquid residue distilled: bp 58-63°C/ 10 mm; n_D^{19} 1.3990, yield 7.2 g (86%). Its ^{1}H-NMR spectrum (Varian T-60 at 60 MHz) did not show olefinic protons but three sets of signals at δ 1.00 (d, CH_3), 1.7 (m, CHDMe) 2.43 (d, CHDCO).

Dideuterobutyryl chloride

Dideuterobutyric acid (7 g) and 15 ml of thionyl chloride were heated at reflux temperature for 1 h. Excess thionyl chloride was removed under low vacuum at room temperature and the liquid residue distilled through at short Vigreux column, bp 95-100°C/760 mm; yield 5.4 g (55%).

Dideuterobutyryldeacetylcolchicine (5)

To 200 mg (0.56 mmol) of 2 in 10 ml of dichloromethane and 0.17 ml of triethylamine was added dropwise under stirring a solution of 100 mg (0.8 mmol) of dideuterobutyryl chloride in 5 ml of dichloromethane. After standing overnight, the solution was washed with water (4 x 3 ml), dried (Na_2SO_4), concentrated and the residue chromatographed on a column of silica gel-60, using, as eluent, chloroform with 1% methanol (100 ml) followed by chloroform with 1.75% methanol and 0.25% ammonia. The main compound was 5 (152 mg), a homogeneous amorphous solid, melting range 126-135°C with gas evolution $[\alpha]_D^{20} = -146.1°$ (c, 1.36, $CHCl_3$).

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