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Antitumor Agents. Part 186:† Synthesis and Biological Evaluation of Demethylcolchiceinamide Analogues as Cytotoxic DNA Topoisomerase II Inhibitors

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Abstract—Demethylation of colchiceinamide (**2**) and its analogues (**3–10**) afforded a novel class of mammalian DNA topoisomerase II inhibitors (**2a–10a**) without displaying tubulin inhibitory activity. All target compounds inhibited the catalytic activity of topoisomerase II at drug concentrations at 100 μM. An in vitro cytotoxicity assay indicated that compounds **3a** and **8a** were strong and tissue-selective cytotoxic agents against the MCF-7 breast cancer cell line ($IC_{50} = 0.36$ and 0.48 μg/mL, respectively) and the CAKI-1 renal cancer cell line ($IC_{50} = 0.72$ and 0.96 μg/mL, respectively).

Introduction

Colchicine (**1**) (Fig. 1), a naturally occurring colchicum alkaloid, is a well known tubulin toxin.[2] It has been used clinically in the treatment of gout, familial Mediterranean fever, and liver cirrhosis.[3] However, its effects against tumors were only observed at toxic or near toxic levels.[4] Numerous colchicine analogues have been discovered through extensive structural modifications.[5–7] Colchiceinamide (**2**) (Fig. 1), with an amino group instead of a methoxy group at the C(10) position, displayed a more pronounced antitumor effect and less toxicity than colchicine at an optimal dose.[8]

Our previous studies demonstrated that colchicine analogues can be targeted to a different intracellular protein, DNA topoisomerase II (topo II), through exhaustive A-ring demethylation.[9,10] The type II topoisomerase is capable of interconverting DNA topoisomers by passing an intact helix through a transient double-stranded break in the DNA backbone.[11] Because of its critical role in cellular functions such as transcription, replication, and chromosome segregation at mitosis,[12] topoisomerase II is the primary cellular target for many widely prescribed antibiotics and anticancer drugs.[13] Unlike topoisomerase poisons (e.g. VP-16) that induce protein-linked DNA breakage, demethylcolchicine analogues appear to be "non-cleavable" inhibitors that do not induce covalent enzyme–DNA complexes intracellularly and may not be cross-resistant with conventional agents used in clinical therapy.[1,9] Our recent investigation further identified the trihydroxy A-ring as a critical pharmacophore for maximal topoisomerase II inhibitory activity.[1] We also found that B ring side chain modifications are tolerated leading to compounds showing topo II enzyme inhibition with improved cytotoxicity.

As a part of our ongoing effort to understand the structure–activity relationship (SAR) of this compound class and develop cytotoxic topo II inhibitors with

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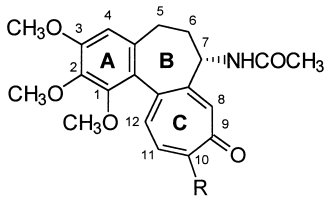


Colchicine **1**: R = OCH_3

Colchiceinamide **2**: R = NH_2

Figure 1. Structures of colchicine and colchiceinamide.

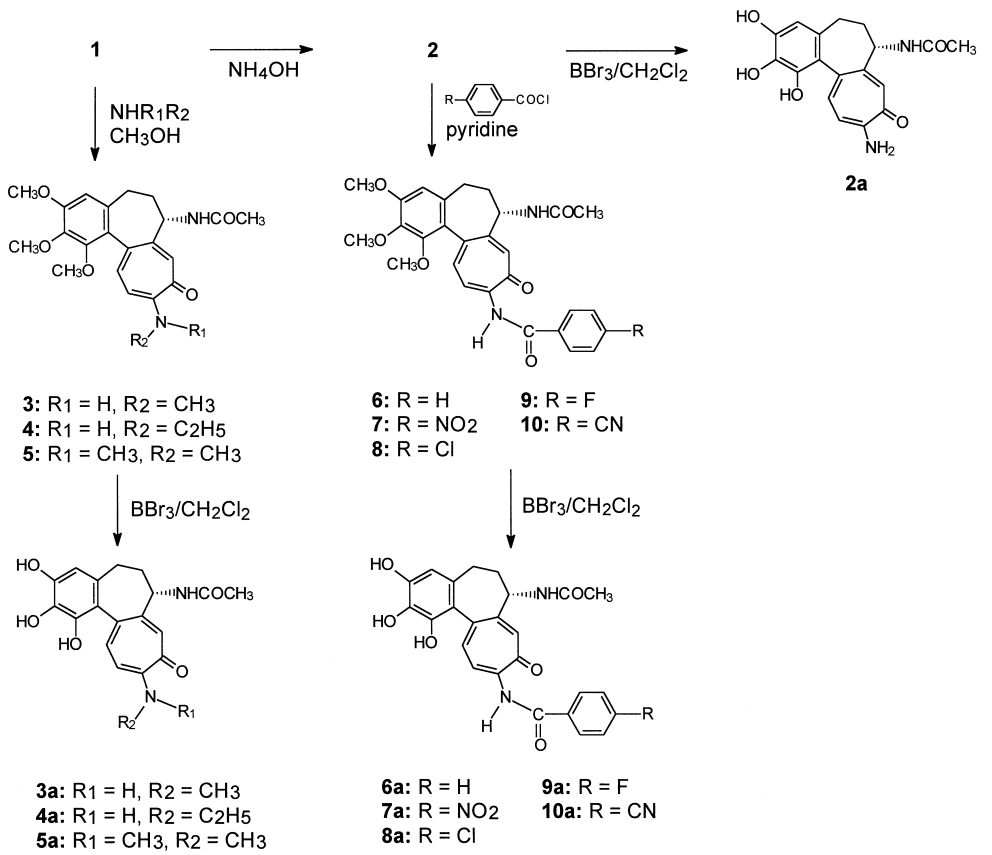
clinical potential, we modified the C-ring amino group of colchiceinamide in conjunction with complete A-ring demethylation. In this paper, we report the synthesis and biological evaluation of additional novel DNA topoisomerase II inhibitors, demethylcolchiceinamide derivatives **2a–10a**. Some displayed potent and selective cytotoxic activity with an antitumor spectrum distinct from that of colchicine.

Chemistry

The general procedure used for preparing target compounds **2a–10a** is outlined in Scheme 1. Compounds **2–10** were prepared according to the methods developed earlier.[14,15] Colchiceinamide was readily available from **1** by treatment with ammonium hydroxide. N-alkylated colchiceinamides **3–5** were obtained by heating **1** and alkylamines in a sealed vessel. Condensation of **2** with various aroyl chlorides gave N-aroyl-colchiceinamides **6–10**. Exhaustive demethylation of **2–10** with excess boron tribromide in dichloromethane at room temperature afforded corresponding demethylated compounds **2a–10a**.

Biological Results and Discussion

In a preliminary in vitro topo II inhibitory assay, all target compounds completely inhibited enzyme-catalyzed



Scheme 1. Synthesis of demethylcolchiceinamides **2a**–**10a**.

DNA unknotting at drug concentrations of 100 μM (Table 1). However, their fully methylated analogue, compound **2**, was inactive. Under the same concentrations, DNA topoisomerase I activity was not influenced by the test compounds. Exhaustive demethylation abolished their antimitotic activity because inhibition of tubulin polymerization was not observed at drug concentrations as high as 40 μM. Our current data shows that the modification of the tropolonic amine did not make an obvious difference in topo II inhibition.

Table 2 summarizes the ability of **1**, VP-16, and **2a–10a** to inhibit tumor cell growth. After three days of drug treatment, the EC_{50} value was determined as the compound concentration that caused a 50% reduction in absorbance at 562 nm relative to untreated cells using an SRB assay.[16] Compound **2a** with a free amino group was inactive. The other compounds containing variable side chains showed marginal to potent cytotoxicity. Generally, demethylcolchiceinamides were less active than **1** and VP-16 in inhibiting tumor growth. However, the breast cancer cell line MCF-7 was an exception. Colchicine produced a plateau dose response in this cell line with maximum growth inhibition of 45%. Similarly, VP-16 only produced 34% inhibition in this cell line. Interestingly, compounds **3a** and **8a** displayed significant cytotoxic activity against MCF-7 cells with EC_{50} values of 0.36 μg/mL and 0.48 μg/mL, respectively. In addition, their growth inhibitory effects against the renal cancer cell line CAKI-1 were comparable to those of **1** and VP-16. Compounds **3a** and **8a** were only weakly cytotoxic toward KB and A-549 cells and were inactive against SK-MEL-2. Thus, **3a** and **8a** exhibited tissue-selective cytotoxicity and a different antitumor pattern from that of **1**.

Table 1. Evaluation of demethylcolchiceinamides as inhibitors of tubulin and DNA topoisomerase II in vitro

Compound	% inhibition of DNA		ITP[c] IC_{50} (μM)
	Topo II activity (100 μM)[a]	Topo I activity (100 μM)[b]	
2	0		1–4
2a	100	0	> 40
3a	100	0	> 40
4a	100	0	> 40
5a	100	0	> 40
6a	100	0	> 40
7a	100	0	> 40
8a	100	0	> 40
9a	100	0	> 40
10a	100	0	> 40
VP–16	100	0	IN[d]

[a] Measured as ATP-dependent unknotting of P4 DNA compared to enzyme and DNA control reactions. VP–16 was used as a positive control. No active compound displayed measurable enzyme inhibition when tested at 10 μM concentration.
[b] Measured as ATP-independent relaxation of supercoiled plasmid DNA compared to enzyme and DNA control reaction. Camptothecin at 100 μM served as the positive control.
[c] Concentrations which inhibit tubulin polymerization by 50% compared to the control.
[d] In active at 100 μM.

In summary, demethylcolchiceinamide analogues are a novel class of selective DNA topoisomerase II inhibitors without antimitotic activity. The data obtained from an in vitro tumor growth inhibition assay revealed that **3a** and **8a** displayed an improved cytotoxic profile (less toxic and more selective) compared to **1**. In vitro and intracellularly protein-linked DNA complex formation assays are currently ongoing in order to examine their possible mechanism of action as cytotoxic topo II inhibitors.

Experimental

Chemistry

Melting points were determined on a Fisher-Johns melting apparatus and are uncorrected. Optical rotations were determined with a DIP-1000 polarimeter. ^1H NMR spectra were recorded on a Bruker AC-300 spectrometer. The chemical shifts are presented in terms of ppm with Me_4Si as the internal reference. Mass spectra were measured by NIH. MCI gel CHP-20P was purchased from Supelco. Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA.

General procedure for synthesis of demethylated colchiceinamides (2a–10a). To a solution of starting material in anhydrous CH_2Cl_2 was added dropwise a 1 M solution of boron tribromide in CH_2Cl_2 (molar ratio of 1/10) under ice cooling. The reaction mixture was stirred at room temperature for 24 h. After cooling the reaction mixture in an ice bath, methanol (20 mL) was added dropwise. The solution was refluxed for 2 h, then the solvent was removed in vacuo. The residue was purified by MCI Gel CHP-20P column chromatography using H_2O and MeOH (50%, v/v) as eluents.

Tridemethylcolchiceinamide (2a). Yield 27.7% (starting from 210 mg of **2**); amorphous; $[\alpha]_D^{25}$ −273.6° (*c* 0.13, MeOH); ^1H NMR (CD_3OD) δ 2.0 (3H, s, $COCH_3$), 2.17–2.40 (4H, m, H-5,6), 4.52 (1H, m, H-7), 6.30 (1H, s, H-4), 7.11 (1H, d, J = 11.3 Hz, H-11), 7.28 (1H, s, H-8), 7.45 (1H, d, J = 11.3 Hz, H-12); Anal. calcd (found) for $C_{18}H_{18}N_2O_5 \cdot 1¼H_2O$: C, 59.25 (59.45); H, 5.66 (5.68); N, 7.68 (7.28).

Tridemethyl-N-methyl-colchiceinamide (3a). Yield 81.5% (starting from 171.7 mg of **3**); amorphous; $[\alpha]_D^{25}$ −167.7°

Table 2. Inhibition of in vitro tumor cell growth[a,b] by demethylcolchiceinamide analogues

Compound	Cytotoxicity EC_{50} (μg/mL)[c]				
	KB	A549	MCF-7	CAKI-1	SK-MEL-2
1	0.002	0.022	> 0.4 (33–45)[d]	0.4	0.008
VP–16	0.200	1.23	3.82 (34)	1.40	ND[e]
2a	> 13.7 (0)[f]	> 13.7 (0)	> 13.7 (23)	> 13.7 (5)	> 13.7 (18)
3a	11.7	14.2	0.36	0.72	14.2
4a	13.7	> 4.8 (31)	12.2	> 14.8 (0)	> 14.8 (0)
5a	13.7	> 14.8 (39)	10.7	> 14.8 (13)	> 14.8 (0)
6a	> 17.8 (48)	> 17.8 (27)	16.1	> 17.8 (0)	> 17.8 (0)
7a	17.2	18.6	1.47	ND	> 19.6 (6)
8a	17.3	> 0.28 (24–32)	0.48	0.96	> 19.2 (24)
9a	18.1	> 0.6 (24–32)	15.3	> 18.6 (23)	> 18.6 (11)
10a	16.9	> 18.8 (39)	13.6	> 18.8 (13)	> 18.8 (0)

[a] Data obtained from our in-house in vitro disease-oriented human tumor cell screen.
[b] KB, epidermoid carcinoma of the nasopharynx; A549, lung carcinoma; MCF-7, breast adenocarcinoma; CAKl-1, kidney carcinoma; SK-MEL-2, malignant melanoma.
[c] EC_{50} is the concentration of compound that causes a 50% reduction in absorbance at 562 nm relative to untreated cells using SRB assay.
[d] Plateau dose response observed.
[e] ND = not determined.
[f] Observed percent inhibition in parentheses.

(*c* 0.12, MeOH); ^{1}H NMR (pyridine-d_5) δ 2.0 (3H, s, $COCH_3$), 2.24–2.50 (4H, m, H-5,6), 2.75 (3H, d, J = 5.4 Hz, NCH_3), 5.37 (1H, m, H-7), 6.34 (1H, d, J = 11.2 Hz, H-11), 6.82 (1H, s, H-4), 7.82 (1H, d, J = 11.2 Hz, H-12), 7.94 (1H, s, H-8), 9.27 (1H, d, NHCO); Anal. calcd (found) for $C_{19}H_{20}N_2O_5 \cdot H_2O$: C, 60.95 (61.05); H, 5.92 (5.89); N, 7.48 (7.12).

Tridemethyl-N-ethyl-colchiceinamide (4a). Yield 70% (starting from 100 mg of **4**) amorphous; $[\alpha]_D^{25}$ −278.9° (*c* 0.09, MeOH); ^{1}H NMR (pyridine-d_5) δ 1.0 (3H, t, CH_3-10), 2.0 (3H, s, $COCH_3$), 2.20–2.40 (4H, m, H-5,6), 3.0 (2H, m, CH_2-10), 5.37 (1H, m, H-7), 6.43 (1H, d, J = 11.2 Hz, H-11), 6.63 (1H, s, H-4), 7.83 (1H, d, J = 11.2 Hz, H-12), 7.97 (1H, s, H-8), 9.30 (1H, d, NHCO); Anal. calcd (found) for $C_{20}H_{22}N_2O_5 \cdot 2\frac{1}{2}CH_3CO_2C_2H_5$: C, 61.00 (60.55); H, 7.17 (7.18); N, 4.74 (5.22).

Tridemethyl-N-dimethyl-colchiceinamide (5a). Yield 66% (starting from 156.8 mg of **5**); amorphous; $[\alpha]_D^{25}$ +1.1° (*c* 0.7, MeOH); ^{1}H NMR (pyridine-d_5) δ 1.53 (3H, s, $COCH_3$), 1.6–2.2 (4H, m, H-5,6), 2.52 (6H, s, $N(CH_3)_2$), 5.02 (1H, m, H-7), 6.01 (1H, d, J = 11.2 Hz, H-11), 6.43 (1H, s, H-4), 7.31 (1H, d, J = 11.2 Hz, H-12), 7.35 (1H, s, H-8), 8.79 (1H, d, NHCO); Anal. calcd (found) for $C_{20}H_{22}N_2O_5 \cdot H_2O$: C, 61.85 (61.57); H, 6.23 (6.18); N, 7.21 (6.86).

Tridemethyl-N-benzoyl-colchiceinamide (6a). Yield 12.8% (starting from 488 mg of **6**); amorphous; $[\alpha]_D^{25}$ −291.4° (*c* 0.73, MeOH); ^{1}H NMR (CD_3OD) δ 2.0 (3H, s, $COCH_3$), 1.88–2.44 (4H, m, H-5,6), 4.5 (1H, m, H-7), 6.29 (1H, s, H-4), 7.49 (1H, s, H-8), 7.53 (2H, d, J = 7.2 Hz, H-3′,5′), 7.59 (1H, d, J = 11.0 Hz, H-11), 7.96 (2H, d, J = 7.2 Hz, H-2′,6′), 9.13 (1H, d, J = 11.0 Hz, H-12); Anal. calcd (found) for $C_{25}H_{22}N_2O_6 \cdot 1\frac{1}{2}H_2O$: C, 63.42 (63.42); H, 5.32 (5.46); N, 5.92 (5.77).

Tridemethyl-N-(4′-nitrobenzoyl)-colchiceinamide (7a). Yield 54.2% (starting from 569 mg of **7**) amorphous; $[\alpha]_D^{25}$ −138.2° (*c* 0.85, acetone); ^{1}H NMR (CD_3OD) δ 2.1 (3H, s, $COCH_3$), 2.19–2.42 (4H, m, H-5,6), 4.53 (1H, m, H-7), 6.27 (1H, s, H-4), 7.47 (1H, s, H-8), 7.58 (1H, d, J = 10.9 Hz, H-11), 7.63 (1H, s, H-4′), 8.16 (2H, d, J = 8.6 Hz, H-3′,5′), 8.37 (2H, d, J = 8.5 Hz, H-2′,6′), 9.10 (1H, d, J = 10.9 Hz, H-12); Anal. calcd (found) for $C_{25}H_{21}N_3O_8 \cdot H_2O$: C, 58.94 (58.83); H, 4.55 (4.67); N, 8.25 (8.07).

Tridemethyl-N-(4′-chlorobenzoyl)-colchiceinamide (8a). Yield 25% (starting from 522.7 mg of **8**); amorphous; $[\alpha]_D^{25}$ −212° (*c* 0.25, MeOH); ^{1}H NMR (CD_3OD) δ 2.0 (3H, s, $COCH_3$), 1.84–2.28 (4H, m, H-5,6), 4.54 (1H, m, H-7), 6.29 (1H, s, H-4), 7.49 (1H, s, H-8), 7.57 (2H, d, J = 8.5 Hz, H-3′,5′), 7.60 (1H, d, J = 10.3 Hz, H-11), 7.96 (2H, d, J = 8.5 Hz, H-2′,6′), 9.12 (1H, d, J = 10.3 Hz, H-12); Anal. calcd (found) for $C_{25}H_{21}N_2O_6 \cdot H_2O$: C, 60.19 (60.22); H, 4.65 (4.83); N, 5.61 (5.40).

Tridemethyl-N-(4′-fluorobenzoyl)-colchiceinamide (9a). Yield 12.7% (starting from 506 mg of **9**); amorphous;

$[\alpha]_D^{25}$ −239.1° (*c* 0.88, MeOH); ^{1}H NMR (CD_3OD) δ 1.97 (3H, s, $COCH_3$), 1.85–2.40 (4H, m, H-5,6), 4.53 (1H, m, H-7), 6.26 (1H, s, H-4), 7.26 (2H, d, J=7.9 Hz, H-3′,5′), 7.46 (1H, s, H-8), 7.55 (1H, d, J=10.8 Hz, H-11), 7.98 (2H, d, J=7.9 Hz, H-2′,6′), 9.07 (1H, d, J=10.8 Hz, H-12); Anal. calcd (found) for $C_{25}H_{21}N_2O_6 \cdot 2¼H_2O$: C, 61.79 (61.59); H, 5.29 (4.92); N, 5.76 (5.50).

Tridemethyl-N-(4′-cyanobenzoyl)-colchiceinamide (10a). Yield 20% (starting from 513 mg of **10**); amorphous; $[\alpha]_D^{25}$ −295.9° (*c* 1.2, MeOH); ^{1}H NMR (CD_3OD) δ 1.97 (3H, s, $COCH_3$), 1.8–2.4 (4H, m, H-5,6), 4.5 (1H, m, H-7), 6.25 (1H, s, H-4), 7.45 (1H, s, H-8), 7.54 (1H, d, J=10.9 Hz, H-11), 7.86 (2H, d, J=8.0 Hz, H-3′,5′), 8.05 (2H, d, J=8.0 Hz, H-2′,6′), 9.05 (1H, d, J=10.9 Hz, H-12); Anal. calcd (found) for $C_{26}H_{21}N_3O_6 \cdot 1¼CH_3CO_2C_2H_5$: C, 64.02 (64.07); H, 5.37 (5.55); N, 7.22 (7.55).

DNA topoisomerase II catalytic assay

Inhibition of topoisomerase II catalytic activity was examined using the standard P4 DNA unknotting assay.[17] The reaction mixture (20 μL) which contained 50 mM Hepes (pH 6.7), 50 mM KCl, 100 mM NaCl, nuclease-free bovine serum albumin (50 μg/mL), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ATP, P4 DNA substrate (0.26 μg), 1.25 U enzyme was incubated with or without drugs as the initial stage of screening for activity. The reaction mixture was incubated at 37 °C for 30 min and terminated by adding a stop solution (2% SDS, 20% glycerol and 0.05% bromophenol blue). These samples were loaded onto a 1% agarose gel and electrophoresed at 50 V overnight with an eletrophoresis buffer which contained 90 mM Tris boric acid (pH 8.3) and 2.5 mM EDTA. At completion, the gel was stained in a 0.5 mg/mL of ethidium bromide. Then a photograph (Polaroid Type 667 film) was taken of the DNA bands visualized with fluorescence induced by a long wavelength UV-lamp. One unit was defined as the amount of enzyme which converted 50% of the knotted DNA on the gel to the unknotted form in 30 min.

Cytotoxicity assay

For testing, samples diluted in culture medium immediately prior to use were prescreened at 4, 2, and 1 μM versus HTCL. HTCL panel cultured in RPMI-1640, FBS 10% (v/v), and kanamycin (100 μg/mL). Sulforhodamine B assays, triplicate dose treatment and three day exposure were standard procedures. Based on prescreening results, compounds were further tested versus HTCL to establish EC_{50} values.

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