BIOORGANIC CHEMISTRY 6, 453–463 (1977)

Studies Related to Antitumor Antibiotics
X. Reactions of Maytansine and its Analogs with DNA *in Vitro*[1]

J. WILLIAM LOWN AND KRISHNA C. MAJUMDAR

Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2

ALBERT I. MEYERS AND ANN HECHT

Department of Chemistry, Colorado State University, Fort Collins, Colorado 80521

Received February 7, 1977

The antileukemic agent maytansine and certain synthetic carbinolamide analogs which also show antineoplastic activity rapidly alkylate nucleic acids in a reaction which is promoted by acidic conditions. Alkylation is evidenced by the heat-induced strand scission of alkylated covalently closed circular DNA detected by ethidium fluorescence assay. Alkylation of poly(d[^{14}C]G)·poly(d[^{3}H]C) by carbinolamide at 37°C is accompanied by neither depurination nor depyrimidation. The reaction of maytansine with DNA is interpreted as acid-induced dehydration of the carbinolamide moiety to an azomethine lactone and subsequent attack on this species by nucleophiles on the bases of DNA. The observed lack of reactivity and the low alkylating ability of two different analogs, both of which undergo dehydration very slowly, and a third analog, where loss of the alcohol is resisted, are in accord with this interpretation and the known loss of antileukemic activity of maytansine upon conversion to the 9-ether derivative. Certain carbinolamides at a concentration of 9×10^{-5} M have no effect on the rate of *Escherichia coli* DNA polymerase I catalyzed synthesis of duplex DNA on denatured calf thymus DNA template.

INTRODUCTION

The maytansinoids are a group of structurally related ansa macrolides, isolated from the East African plant species *Maytenus serrata* and *Colubrina*, which are of considerable clinical interest because of their high potency against P388 lymphocytic leukemia (*1–5*). Maytansine (Fig. 1), the most thoroughly studied representative of this class (and which has been described as the most promising antineoplastic agent isolated from plant sources (*6*)), also exhibits inhibitory activity against solid murine tumors, e.g., the B16 melanocarcinoma and Lewis lung carcinoma, as well as activity *in vitro* against cells derived from human carcinoma of the nasopharynx, KB (*1, 3, 5*) and is currently undergoing clinical trials (*7*). Maytansine is a highly active inhibitor of cell division (*8, 9*) and of transformation of mouse cell cultures infected with murine sarcoma virus (*10*). The tumor inhibitory activity of the maytansinoids appears to be associated with the selective alkylation of nucleophiles in growth regulatory biological macromolecules (*4*) and the thiol groups of key enzymes have been suggested as possible cell target sites (*4*). However, the recent observation in a study of the action of

[1] This research was supported by grants to J.W.L. from the National Cancer Institute of Canada, the National Research Council of Canada, and by the Department of Chemistry, University of Alberta, and to A.I.M. from the National Institutes of Health, National Cancer Institute, Washington, D.C.

ISSN 0045–2068

maytansine against the growth of murine leukemia cells that of the three principal macromolecular synthetic processes (DNA, RNA, protein), DNA synthesis was inhibited to the greatest extent, suggested the nucleic acids as alternative cell target sites for maytansine action (*9, 11*).

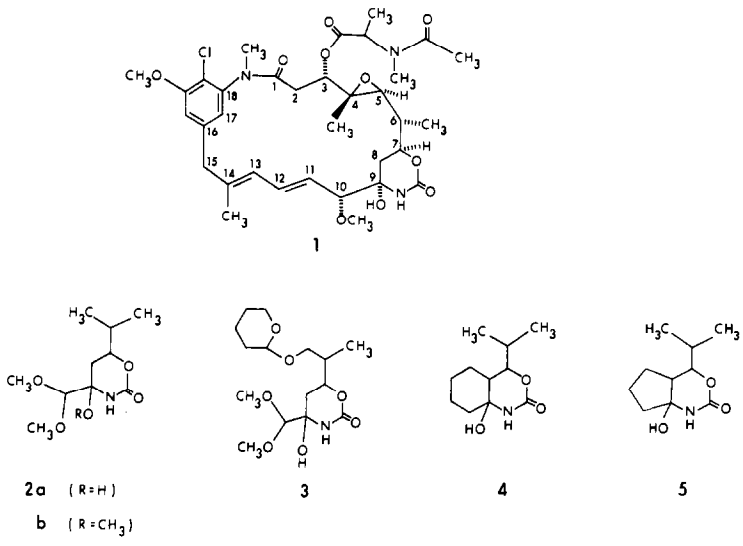

FIG. 1. The structure of maytansine **1** and the synthetic carbinolamide analogs **2–5**.

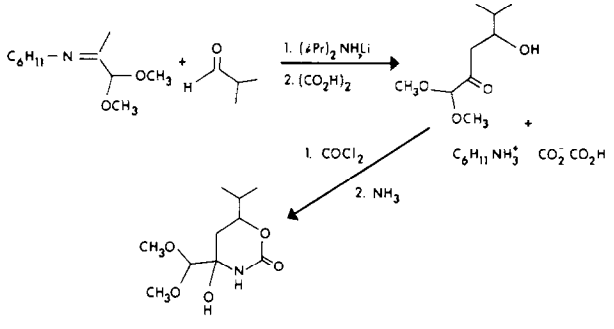

FIG. 2. Route of synthesis of model carbinolamides.

We wished to test the hypothesis that the carbinolamide moiety, which has been shown to be necessary for antitumor activity, could serve as a potential azomethine lactone (by dehydration) and therefore act as a powerful alkylating agent for cell target sites. Accordingly, a number of model carbinolamides **2a**, **2b**, **3**, **4**, and **5** were synthesized by the general procedure given in Fig. 2. We report an examination of the chemical reactions of maytansine and these synthetic analogs of the active carbinolamide moiety with nucleic acids.

[2] Abbreviations used: CLC, covalently linked complementary; CCC, covalently closed circular; OC, open circular.

EXPERIMENTAL

Materials

Maytansine was kindly supplied by (the late) Professor S. M. Kupchan, University of Virginia. Ethidium bromide was purchased from Sigma Chemicals. PM2-CCC[2] and calf-thymus-DNAs were obtained as described previously (*12*). *Escherichia coli* DNA polymerase I was obtained from Dr. A. R. Morgan, University of Alberta.

All melting points were taken on a Buchi melting-point apparatus and are uncorrected. All boiling points are uncorrected. Analyses were carried out by Midwest Microlab., Ltd. The infrared spectra were recorded on a Perkin–Elmer Model 267 spectrometer. The proton magnetic resonance spectra were recorded by means of JEOLCO, 100 MHz and Varian T-60 spectrometers, with tetramethylsilane as internal reference. The mass spectra were obtained with an AEI-MS12 high resolution spectrometer.

Dimethylacetal of Pyruvaldehydecyclohexylimine

In a 100-ml round-bottomed three-necked flask cooled to −14°C (NH_4Cl, ice) and equipped with a nitrogen inlet, serum cap, and magnetic stirrer was placed 24.2 ml (0.2 mol) of freshly distilled cyclohexylamine. Dimethylacetal of pyruvaldehyde, 23.8 ml (0.2 mol), was added slowly with stirring and the reaction mixture was stirred for 1 hr. Sodium sulfate (4 g) was added and stirring continued for a further 35 min. The solution was filtered, the residue was washed with 50 ml of ether, and the organic phase was dried over anhydrous potassium carbonate. After filtering and evaporation of the solvent a yellow liquid remained which afforded the imine as a colorless oil, 37.02 g (93% yield), upon distillation, bp 64–66°C (0.4 mm). The imine was stable when stored under refrigeration: pmr (CCl_4) δ 1.5 (*m*, 14), 3.35 (*s*, 6), 4.15 (*s*, 1).

4-Hydroxy-2-keto-5-methylhexane-1-aldehyde Dimethyl Acetal

A 100-ml dry round-bottomed three-necked flask equipped with a nitrogen inlet, serum cap, and magnetic stirrer was evacuated with a vacuum pump and then filled with nitrogen (under 0.1 atm of positive pressure from a mercury trap). The procedure was repeated twice; then diisopropylamine, 8.16 ml (58 mmol), in 60 ml of dry tetrahydrofuran was added and the solution was cooled to 0°C. *n*-Butyl lithium solution, 24.2 ml (58 mmol), was added slowly and the solution was stirred for 30 min. The imine above, 10.6 g (53 mmol), was added in 10 ml of dry tetrahydrofuran and the anion was allowed to form during 30 min. The reaction mixture was cooled to −78°C (acetone, CO_2), freshly distilled isobutyraldehyde, 4.8 ml (53 mmole), was added, and the mixture was stirred for 1 hr at −78°C. The reaction mixture was allowed to reach ambient temperature over a period of 4.5 hr. The reaction was quenched with 200 ml of saturated oxalic acid solution, extracted with methylene chloride (3× 100 ml), and the combined organic extracts were washed with saturated sodium chloride solution and then dried over anhydrous potassium carbonate. The solvent was removed from the filtered solution and the residual oil was distilled, bp 67–69°C (0.3 mm), to afford the title acetal as a clear liquid, 7.64 g (78% yield). pmr (CCl_4) δ 1.0 (*d*, 6), 1.7 (*m*, 1), 2.7 (*d*, 2), 3.15 (*s*, 1), 3.40 (*s*, 6), 3.9 (quart, 1), 4.5 (*s*, 1).

Carbinolamides **2a, 2b**

In a 150-ml round-bottomed three-necked flask equipped with a nitrogen inlet, serum cap, and magnetic stirrer was placed the above β-hydroxyketone, 8.11 g (40.8 mmol); dry pyridine, 6 ml (76 mmol); and phosgene, 32.3 ml (12.5% benzene solution, 40.8 mmol). The mixture was stirred for 2 hr under nitrogen at room temperature. In a separate flask, 15 ml of anhydrous methanol (distilled from magnesium) was cooled to 0°C and approximately 3 g of liquid ammonia was added. The original reaction mixture was cooled to −50°C (acetone, CO_2) and the methanol–ammonia mixture was added. Stirring was continued for 4 hr as the reaction vessel returned to ambient temperatures. Benzene (100 ml) was added and the solid precipitate was collected. The solid was suspended in benzene and filtered again. The combined filtrates were concentrated to a red oil which was heated under reflux in ether on a steam bath. The resulting solid was recrystallized from carbon tetrachloride giving carbinolamide **2a**, 1.30 g (14% yield), mp 110.5–111°C.

Anal. Calcd for $C_{10}H_{19}NO_5$: C, 51.49; H, 8.21; N, 6.01. Found: C, 51.47; H, 8.13; N, 6.10. pmr ($CDCl_3$) δ 1.0 (pair of *d*, 6, $J = 8$ Hz), 1.87 (*m*, 3), 3.41 (*s*, 1), 3.6 (pair of *s*, 6), 4.36 (*m*, 2), 6.18 (*s*, 1). ir ν_{max}(film) 3540, 3410 (OH, NH), 1700 cm^{-1} (>C=O).

A solution of 25 mg of carbinolamide **2a** and 0.1 mg of *p*-toluenesulfonic acid in 1 ml of dry methanol was maintained at 50° for 30 min. The methanol was removed and the remaining oil was taken up in 2 ml of methylene chloride and passed through a 6 × 1.2-cm column of basic alumina. Removal of the solvent from the eluate gave carbinolamide **2b**, 20 mg (70% yield), as a clear oil. pmr ($CDCl_3$) δ 1.0 (pair of *d*, $J = 7$ Hz, 6, $-CH(CH_3)_2$); 1.9 (*m*, 3). 3.35. 3.5, 3.55 (*s*, three each OCH_3), 4.28 (*m*, 1, $CH(CH_3)_2$), 6.85 (*s*, 1*H*, NH).

2(1'-Hydroxy-2'-methylpropyl)cyclohexanone

In a 250-ml round-bottomed three-necked flask equipped with a magnetic stirrer, nitrogen inlet tube, and serum cap was placed diisopropylamine, 6.8 ml (48.5 mmol), in 50 ml of dry tetrahydrofuran. The system was cooled to −78°C and *n*-butyl lithium, 19.4 ml (48.5 mmol), was added with stirring and the mixture was stirred for 30 min. Freshly distilled cyclohexanone, 5.0 ml (48.5 mmol), was added in 10 ml of dry tetrahydrofuran and stirring continued for 30 min. Freshly distilled isobutyraldehyde, 4.40 ml (48.5 mmol), taken up in 6 ml of dry tetrahydrofuran, was added to the mixture and stirred for a further 30 min at −78°C. The reaction was then quenched with 100 ml of water. The aqueous phase was extracted with ether (3 × 50 ml). The combined ether layers were washed with 100 ml of saturated sodium chloride solution and then dried over potassium carbonate. The filtered solution upon concentration gave the substituted cyclohexanone as an oil, 7.95 g (96.3% yield). Attempts at further purification resulted in quantitative elimination. pmr (CCl_4) δ 0.90 (*d*, 6), 1.18–2.66 (*m*, 10), 3.37 (*m*, 2). ir ν_{max}($CHCl_3$) 3510 (OH), 1690 cm^{-1} (CO).

Carbinolamide **4**

2(1'-Hydroxy-2'-methylpropyl)cyclohexanone, 1.00 g (5.92 mmol), was placed in a 25-ml three-necked bottomed flask equipped with a nitrogen inlet tube, a serum cap, and a Dewar additional funnel. Dry pyridine, 0.5 ml (6.3 mmol), and 6 ml of dry ether were added and the mixture was stirred under nitrogen. The system was cooled to 0°C and

phosgene, 5.0 ml (12.5% benzene solution, 6.3 mmol), was introduced. Within 5 min the white slurry was cooled to −50°C (acetone, CO_2); then gaseous ammonia was allowed to condense on the Dewar addition funnel (filled with acetone, CO_2 −78°C). When an excess of liquid ammonia had been added the reaction mixture was stirred at −50°C for 1 hr; then the reaction was quenched with 25 ml of water. The aqueous phase was extracted with ether (3 × 20 ml) and the combined organic extracts were washed with saturated sodium chloride solution and dried over sodium sulfate. The filtered solution upon concentration gave a yellow oil which crystallized. Recrystallization from ether afforded **4** as white crystals, mp 138–138.5°C in 15% yield.

Anal. Calcd for $C_{11}H_{19}NO_3$: C, 61.95; H, 8.98; N, 6.57. Found: C, 62.12; H, 8.84; N, 6.72. pmr ($CDCl_3$) δ 0.93 (pair of *d*, 6, $J = 8$ Hz), 1.2–3.0 (*m*, 10), 4.66 (*s*, 1), 5.0 (overlapping doublets, 1, $J = 8$ and 7 Hz.)

2(1′-Hydroxy-2′-methylpropyl)cyclopentanone

This compound was prepared as described above for 2(1′-hydroxy-2′-methylpropyl)cyclohexanone. Thus lithium diisopropylamide was formed from diisopropylamine, 15.8 ml (0.11 mol), and *n*-butyllithium, 45.8 ml (0.11 mol of 2.4 *M* solution), in 100 ml of dry tetrahydrofuran. Freshly distilled cyclopentanone, 10 ml (0.11 mol), was added and after stirring for 30 min at −78°C, isobutyraldehyde, 9.97 ml (0.11 mol), was added. The work-up was followed as described previously for the cyclohexanone analog to give the crude β-hydroxyketone, 16.4 g (95.3% yield). Attempted purification by distillation resulted in quantitative elimination. pmr ($CDCl_3$) δ 1.0 (*d*, 6), 1.12–2.6 (*m*, 10), 3.55 (*m*, 1), 4.0 (*s*, OH). ir ν_{max}(film) 3490 (OH), 1715 cm^{-1} (>C=O).

Carbinolamide **5**

2(1′-Hydroxy-2′-methylpropyl)cyclopentanone, 5.02 g (32.3 mmol), 3.22 ml of dry pyridine, and 30 ml of dry tetrahydrofuran were combined as described above for the preparation of **4**. Phosgene, 25.3 ml (32 mmol), was added to the cooled (0°C) system. At −50°C, an excess of liquid ammonia was introduced and the work-up previously described was followed to give **5** as a white crystalline solid from ether, 0.987 g (15.3% yield), mp 146–147°C.

Anal. Calcd for $C_{10}H_{17}NO_3$: C, 60.28; H, 8.60; N 7.03. Found: C, 60.44; H, 8.82: N, 7.27. pmr ($CDCl_3$) δ (1.0 pair of *d*, 6, $J = 7$ Hz), 1.7–2.6 (*m*, 9), 4.7 (*m*, 2, partially disappeared in D_2O, NH). ir (KB_r dis) 3410–3198 (OH, NH), 1722 cm^{-1} (>C=O).

Methods

Fluorescence Determination of Alkylation of PM2-CCC DNA with Maytansine and Carbinolamide Analogs

All measurements were performed on a G. K. Turner and Associates Model 430 spectrofluorometer equipped with a cooling fan to reduce fluctuations in the xenon lamp source. Wavelength calibration was performed as described in the manual for the instrument. One-centimeter-square cuvettes were used. The excitation wavelength was 525 nm and the emission wavelength was 600 nm. The 100× scale of medium sensitivity was generally used and water was circulated between the cell compartment and a thermally regulated bath at 22°C.

The reaction mixtures were buffered to the appropriate pH with potassium phosphate at pH 4.4, 5.8, 6.6, 7.0, 8.6 or with tris hydrochloride at pH 7.0 and 7.5. Alkylation reactions were carried out in a total volume of 150 μl at 37°C. The reaction solution contained approximately 1.60 A_{260} units of PM2-CCC-DNA, (86% CCC, 14% OC) 66 m*M* buffer, e.g., 30 m*M* carbinolamide derivative. At intervals 15-μl aliquots were withdrawn and added to 2 ml of the assay solution which contained 20 m*M* potassium phosphate, pH 11.8, 0.2 m*M* EDTA, and 0.5 μg/ml of ethidium bromide (*13*). The fluorescence was measured using a blank without added sample. The solution was then heat denatured at 96°C on a Temp Blok for 3 min and cooled rapidly in a thermostatted water bath at 22°C for 5 min and the fluorescence was again read. Under these conditions unreacted PM2-CCC-DNA returns to register after heat denaturation because of topological constraints. Alkylated PM2-DNA shows a decrease in fluorescence because of thermally induced cleavage at the site of alkylation. The ratio of the decrease in fluorescence (after heat denaturation and rapid cooling) to that of the control is a measure of the extent of alkylation. In a control experiment it was shown that none of the components interfered with the ethidium fluorescence. The parallel experiments with maytansine were carried out on a 75 to 80-μl scale at 37°C. In this case the reaction solution contained ca. 1.50 A_{260} equivalents of PM2-CCC-DNA, 62.5 m*M* potassium phosphate buffer, pH 6.0, maytansine (4.1 m*M*), and the reactions were carried out as described above for the carbinolamide maytansine analogs. Both maytansine and the carbinolamides were tested in control experiments to see if they caused any single-strand scission of 86% CCC-PM2-DNA by radical-induced cleavage, which would have been apparent by the rise in ethidium fluorescence before heat denaturation as a result of removal of topological constraints (*14*). The carbinolamides **2a**, **3**, and **4** were also tested in a control experiment with 16% CCC-PM2-DNA (84% OC) for evidence of covalent-cross-linking of the OC DNA.

Assay for Possible Depurination of Radioactively Labeled Polynucleotides after Alkylation with Carbinolamide Derivatives

Poly(d[^{14}C]G)·poly(d[^{3}H]C) at a concentration of 0.376 A_{260}/ml was incubated at 37°C in 66 m*M* potassium phosphate buffer, pH 6.0, with various amounts of 100 m*M* carbinolamide derivative **3** (0, 2, 3, 4, 5, 6, and 7 μl) for 40 min in a total volume of 30 μl. Then duplicate samples (10 μl each) were removed, placed on Whatman No. 3 filter disks, washed with 5% trichloroacetic acid three times, then twice with ethanol, dried, and counted.

DNA Synthesis by E. coli DNA Polymerase I on Denatured Calf Thymus DNA Template in the Presence of Carbinolamide **3**

A reaction mixture was prepared containing potassium phosphate, pH 7.5, buffer, 10 m*M* magnesium chloride, 5 m*M* each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytosine triphosphate, and deoxythymidine triphosphate, and 1.02 A_{260} units of denatured calf thymus DNA as template. A 50-μl aliquot of this mixture was treated with 1 μl of an *E. coli* DNA polymerase I solution in 50% glycerol and this served as control. A similar reaction mixture was prepared containing 9×10^{-5} *M* carbinolamide **3** in addition. Both solutions were maintained at 37°C and 10-μl aliquots were withdrawn at timed intervals and assayed for CLC duplex DNA by addition to 2

ml of the standard, pH 11.8, ethidium bromide solution and reading of the fluorescence, immediately and again after heat denaturation at 96°C for 3 min, as described above. The synthesis of new CLC-DNA is revealed by the return of fluorescence after heating since the covalent linker provides a nucleation point for renaturation (*13*).

RESULTS

Maytansine alkylates PM2-CCC-DNA to the extent of 23% within minutes by the addition of a single aliquot at a concentration of 4.1 m*M*. The basis of the assay procedure is that the dye, ethidium bromide, intercalates only into duplex DNA and when it does so suffers a 25-fold enhancement in its fluorescence intensity (*13*). While PM2-CCC-DNA returns to register at pH 11.8 after heat denaturation and cooling because of topological constraints (*12*), alkylated PM2-CCC-DNA upon heating undergoes thermal cleavage at the site of alkylation and the ribosephosphate backbone suffers rapid alkaline cleavage, the strands separate, and a decrease in ethidium fluorescence is observed. The synthetic carbinolamide analogs of the "eastern zone" of maytansine (*15–17*) **2a** and **3** behave in a similar manner while compound **4** does not react at all with DNA and **5** reacts very slowly with DNA. Compound **3** shows a T/C (test to control) value of 132 against the P-388 standard leukemia in rodents (*18*). In addition, while **2a** is very active, conversion to its methyl ether **2b** destroys both its alkylating and antitumor activity.

Alkylation of PM2-DNA by maytansine or carbinolamide is very rapid so that good kinetic curves could not be obtained from a single addition. However, sequential

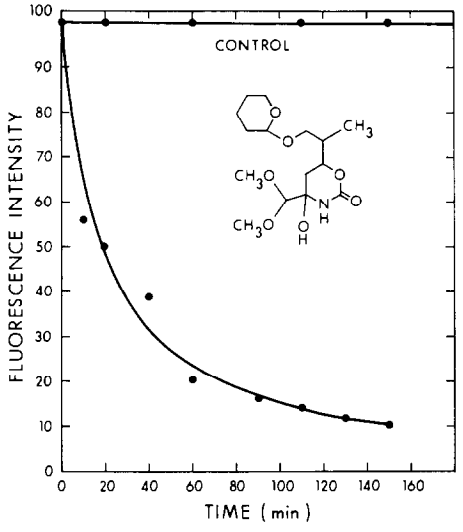

FIG. 3. Alkylation and heat-induced strand scission of PM2-CCC-DNA by sequential treatment with aliquots of the maytansine carbinolamide analog **3**. Reactions were carried out on a scale of 150λ in 66.6 m*M* potassium phosphate, pH 6.0, buffer with a final concentration of PM2-DNA at 1.60 A_{260} units at 20°C. Aliquots (5λ) of a 100 m*M* solution of **3** were added at the intervals 0, 10, 20, 40, and 60 min. The control reaction contained no added carbinolamide. The ordinate represents fluorescence readings after heat denaturation and rapid cooling.

addition of aliquots in the case of carbinolamide **3** gave firm evidence of progressive extents of alkylation up to 90% as evidenced by loss of fluorescence after heat denaturation (Fig. 3) and provided a fair description of the kinetics of the process. The extent of alkylation of DNA by these compounds is favored by acid pH conditions at least in the range 6–8.6. For example, with **2a** at 10 m*M* the extent of alkylation after 10 min of treatment measured by ethidium fluorescence loss thermally induced cleavage at different pH values was 43% (pH 6.0), 24% (pH 7.0), and 8.9% (pH 8.6).

The experiment with the selectively radioactively labeled synthetic polynucleotide (Table 1) shows that alkylation of DNA by carbinolamide **3** at 37°C is accompanied by elimination of neither purine nor pyrimidine bases.

TABLE 1

RADIOACTIVITY ASSAY FOLLOWING ALKYLATION OF Poly(d[^{14}C]G·*Poly*[*d*[^{3}H]C) BY DIFFERENT CONCENTRATIONS OF CARBINOLAMIDE **3**

Carbinolamide (mmol)	^{3}H (cpm)	^{14}C (cpm)	^{14}C/^{3}H ratio
0	1547.5	2300.3	1.48
6.66	1682.5	2496.8	1.48
10	1613.3	2372.4	1.47
13.33	1623.8	2437.8	1.50
16.66	1502.4	2239.7	1.49
20	1609.8	2510.1	1.55
23.33	1530.4	2287.6	1.49

There was no evidence of covalent cross-linking or strand scission of the DNA in treatment with either maytansine or the carbinolamides. Covalent cross-linking of the 84% OC-PM2-DNA would have been apparent since a cross-link would serve as a nucleation point for renaturation after heating at 96°C and rapid cooling resulting in increased return of ethidium fluorescence (*13, 19*). In all cases the percentage return of

TABLE 2

DNA SYNTHESIS MEDIATED BY *E. coli* DNA POLYMERASE I IN THE PRESENCE OF CARBINOLAMIDES **2a**, **3**, AND **4**[a]

	Fluorescence							
	Control		2		3		4	
Time (min)	BH	AH	BH	AH	BH	AH	BH	AH
0	0	0	1.5	0	2	0	1.5	0
30	12	7.5	12	9	10.5	6.5	13	8.5
60	21	13	22	13.5	19	12	22	13.5
90	25	15.5	26	18	25	16	26	17

[a] BH, ethidium florescence at pH 11.8 before heat denaturation and rapid cooling; AH, ethidium fluorescence at pH 11.8 after heat denaturation and rapid cooling.

fluorescence, 16%, equaled that of the control. Similarly, single-strand scission of the sample of supercoiled 86% CCC-PM2-DNA produced by the carbinolamides by a free radical or other mechanism would have been revealed by a characteristic 30% rise in ethidium fluorescence before heat denaturation as a result of release of topological constraints of the DNA (*14*). Again the results in the presence of the carbinolamides equaled that of the control, indicating no such cleavage. The resolution of this assay for strand cleavage is such that one break may be detected in a DNA molecule of 6×10^7 daltons (*14, 20*).

The results in Table 2 show efficient synthesis of CLC-DNA from a denatured calf thymus DNA template with *E. coli* DNA polymerase I. The carbinolamides **2a**, **3**, and **4** at concentrations of 9×10^{-5} *M* had no effect on the rate of synthesis of duplex DNA.

DISCUSSION

The high antineoplastic potency of the maytansinoids coupled with their extremely low isolation yields from natural sources (2×10^{-5}–2×10^{-4}%) make the identification of active fragments of these macrolides which are accessible synthetically, or a viable total synthesis of paramount importance. At least three groups are active currently in this area (*7, 15–17, 21*).

Among the functional groups established from structure–antitumor activity studies of maytansinoids to be necessary for activity, it has been suggested that the aminoester function on the hydrophilic side of the macrolide is involved in the formation of highly selective molecular complexes with growth-regulatory biological macromolecules and that the orientation of the ester may sterically hinder the approach of biological nucleophiles to one side of the molecule (*3*). This action may selectively guide the alkylation of cell target sites by the reactive carbinolamide moiety on the hydrophobic side of the macrolide. This property renders the latter side of maytansine ideally suited to interact with the hydrophobic interior of nucleic acids, and therefore is in accord with the present results and the recent observation noted above that DNA synthesis is strongly inhibited by maytansine in murine leukemia cells (*9, 11*). It is noteworthy that the synthetic carbinolamides (which themselves show significant antineoplastic activity (*18*)) parallel the behavior of the parent maytansine in rapidly alkylating nucleic acids under slightly acidic conditions. This is consistent with the proposed mechanism involving loss of water from the C9–OH of the carbinolamide to form a reactive azomethine lactone intermediate (e.g., **4A**, Fig. 4) which reacts rapidly with biological nucleophiles, e.g., the N-7 position of guanosine. The proposed chemical mechanism receives support from the observed facile acid-catalyzed exchange of the OH group in the carbinolamides for alkoxy and thioalkyl groups (*15*).

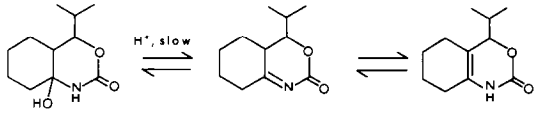

FIG. 4. The slow acid-catalyzed dehydration of the bicyclic carbamate **4**.

The cyclic carbamate **4** does not alkylate DNA and **5** reacts only very slowly. This is evidently due to their slow dehydration rates. In contrast to **2a** or **3** the fused ring system of **4** must flatten out in the transition state before **4A** can be reached and requires more energy than for dehydration of the monocyclic systems. This is related to the problem of generating an exocyclic double bond in cyclohexanes. Additionally the dehydration product of **4** can exist as either **4A** or **4B** (Fig. 4) and the latter will not function as an alkylating agent. Only when **4** is *heated* in benzene with a trace of *p*-toluenesulfonic acid is **4B** formed, although it is quite unstable (*22*). When **4** is dissolved in methanol with a catalytic amount of *p*-toluenesulfonic acid no alkoxyl exchange takes place after 24 hr in contrast to **2a** and **3** which undergo alkoxyl exchange in minutes under these conditions (*22*). In compound **5**, however, since the five-membered ring is closer to coplanarity than the six-membered ring, less energy is required for acid-catalyzed dehydration than for **4** and this is reflected in the slow but measurable alkylation of DNA. At present, with the examples studied there does not appear to be any major effect in the reactivity of the cyclic carbamate as a function of the substituent alpha to OH.

Since tumor cells are characterized by having a lower pH than normal cells due to their high anaerobic glycolytic rate and production of lactic acid (*23, 24*), the observed acid-promoted alkylation of biological macromolecules by maytansine may contribute to the selectivity of the antitumor agent.

Maytansine ethyl ether in which the reactive carbinolamide is no longer available as a potential alkylating function shows no antileukemic activity (*3*). Similarly the methyl ether **2b** is inactive towards DNA because of the difficulty of elimination of the alcohol compared with dehydration of **2a**.

Since it has been suggested that the inhibition of tumor growth by maytansine may be due to selective alkylation of key enzymes which control cell division, the action of the analog **3** on the *E. coli* DNA polymerase I (*25*) catalyzed synthesis of DNA on a calf thymus DNA template was examined by an ethidium fluorescence assay that detects duplex DNA as it is formed. It was found that **3**, **4**, and **5** do not inactivate the enzyme at a concentration of 9×10^{-5} *M*, nor does the concomitant alkylation of the template interfere with the gross synthesis of chemically modified DNA.

In conclusion, the demonstration of the high reactivity of maytansine and its analogs for the chemical modification of nucleic acids, the frequent lethality of such events, and the observed inhibition of DNA synthesis by maytansine, suggests that nucleic acids be considered as possible cell target sites in the mode of action of this drug.

REFERENCES

1. S. M. KUPCHAN, Y. KOMODA, W. A. COURT, G. J. THOMAS, R. M. SMITH, A . KARIM, C. J. GILMORE, R. C. HALTIWANGER, AND R. F. BRYAN, *J. Amer. Chem. Soc.* **94**, 1354 (1972).
2. S. M. KUPCHAN, A. R. BRANFMAN, A. T. SNEDEN, A. K. VERMA, R. G. DAILEY, Y. KOMODA, AND Y. NAGAO, *J. Amer. Chem. Soc.* **97**, 5294 (1975).
3. S. M. KUPCHAN, Y. KOMODA, A. R. BRANFMAN, R. G. DAILEY, AND V. A. ZIMMERLY, *J. Amer. Chem. Soc.* **96**, 3706 (1974).
4. S. M. KUPCHAN, *Fed. Proc.* **33**, 2288 (1974).
5. S. M. KUPCHAN, Y. KOMODA, G. J. THOMAS, AND H. P. J. HINTZ, *J. Chem. Soc. Chem. Commun.* 1065 (1972).

6. S. M. KUPCHAN, *Chem. Eng. News,* **15** (February 28 1974).
7. W. J. ELLIOTT AND J. FRIED, *J. Org. Chem.* **41**, 2469 (1976).
8. S. REMILLARD, L. I. REBHUN, G. A. HOWIE, AND S. M. KUPCHAN, *Science* **189**, 1002 (1975).
9. M. K. WOLPERT-DIFILIPPES, V. H. BONO, R. L. DION, AND D. G. JOHNS, *Biochem. Pharmacol.* **24**, 1735 (1975.
10. T. E. O'CONNOR, C. ALDRICH, A. HADIDI, N. LOMAX, P. OKANO, S. SETHI, AND H. B. WOOD, Proceedings of the 66th Annual Meeting of the American Association of Cancer Research 29 (1975).
11. M. K. WOLPERT-DIFILIPPES, R. H. ADAMSON, R. L. CYSYK, AND D. G. JOHNS, *Biochem. Pharmacol.* **24**, 751 (1975).
12. A. R. MORGAN AND D. E. PULLEYBLANK, *Biochem. Biophys. Res. Commun.* **61**, 346 (1974).
13. A. R. MORGAN AND V. PAETKAU, *Canad. J. Biochem.* **50**, 210 (1972).
14. S. CONE, S. K. HASAN, J. W. LOWN, AND A. R. MORGAN, *Canad. J. Biochem.* **54**, 219 (1976).
15. A. I. MEYERS AND C. C. SHAW, *Tetrahedron Lett.,* 717 (1974).
16. A. I. MEYERS, C. C. SHAW, D. HORNE, L. M. TREFONAS, AND R. J. MAJESTE, *Tetrahedron Lett.,* 1745 (1975).
17. A. I. MEYERS AND R. S. BRINKMEYER, *Tetrahedron Lett.,* 1749 (1975).
18. Results of *in vivo* tests at the National Cancer Institute, NIH (1976).
19. J. W. LOWN, A. BEGLEITER, D. JOHNSON, AND A. R. MORGAN, *Canad. J. Biochem.* **54**, 110 (1976).
20. J. W. LOWN AND K. C. MAJUMDAR, *Canad. J. Biochem.* **55**, 630 (1977).
21. E. J. COREY AND M. G. BOCK, *Tetrahedron Lett.,* 2643 (1975).
22. A. I. MEYERS, unpublished results.
23. T. A. CONNORS, B. C. V. MITCHLEY, V. M. ROSENOER, AND W. C. J. ROSS, *Biochem. Pharmacol.* **13**, 395 (1964).
24. W. C. J. ROSS, *Biochem. Pharmacol.* **8**, 235 (1961).
25. A. KORNBERG, "DNA Synthesis," p. 127. Freeman, San Francisco, 1974.