Synthesis and Antitumor Properties of Bis(quinaldine) Derivatives

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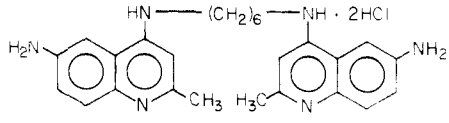
A series of 7-nitro- and amino-*N,N'*-bis(4-quinaldinyl)-α,ω-diaminoalkanes related to the 6-amino derivative 1 was synthesized and tested in the mouse P-388 lymphocytic leukemia screen. Three of the 7-nitro derivatives (**12**, **14**, and **15**) were found to have moderate activity (T/C 140–150%), while other nitro derivatives (11 and **13**) were devoid of any antitumor properties. All five 7-amino compounds (**2–6**) were moderately to strongly active (T/C 134–196%). In addition, binding of amino derivatives **2–6** to DNA was examined by their ability to (1) stabilize DNA to thermal denaturation and (2) inhibit the DNA-dependent RNA polymerase reaction in vitro. T_m data suggest that these compounds bind to DNA and are strong inhibitors of the polymerase reaction (I_{50} = 6–9 × 10^{-6} M).

The bis(quinaldine) (NSC 143057) 1 composed of two substituted quinaldine moieties coupled by an alkyl bridge possesses many of the structural attributes of double or polyintercalating agents.[1-3] Such a molecule might,



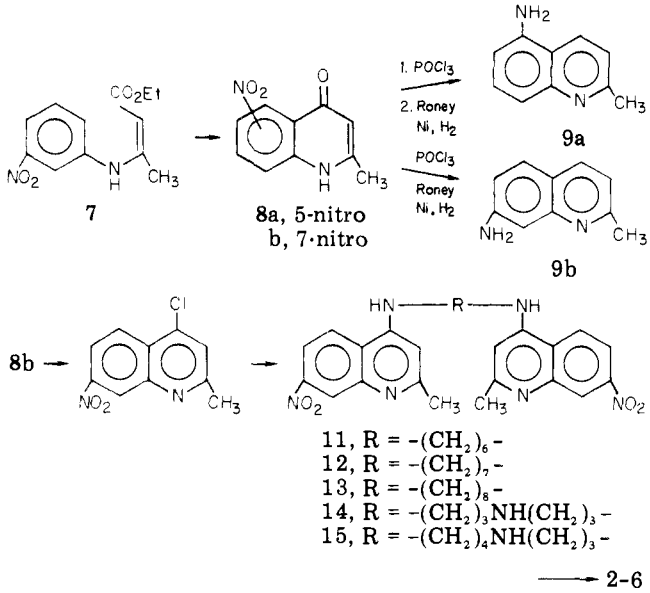
1 (NSC 143057)

therefore, bind at two positions in the same DNA molecule or bind at two different strands of DNA. The overall result of such binding will be inhibition of DNA replication and biosynthesis of RNA. Although 1 was originally synthesized[4] as an agent for the treatment of trypanosomiasis in humans, 1 was shown to have antitumor properties in mouse L1210 leukemia. However, this potential antitumor drug holds little promise of coming to clinical trials due to its poor water solubility which has precluded any meaningful pharmacological studies.

Since the antitumor property of bis(quinaldine) 1 is likely related to its ability to form complexes with nucleic acids, it was believed that structure modification of the parent compound could provide compounds with possibly enhanced affinity to DNA and better antitumor properties. Taking note of proflavin, a compound known for its strong affinity to DNA, it appeared logical that replacement of the 6-amino group of 1 and homologues with a 7-amino group (equivalent to the 6-amino group in proflavin) would provide compounds (**2–4**, Table I) with enhanced binding to DNA and a capability of interfering with the biological function of replication and transcription. Furthermore, in compounds **5** and **6**, additional replacement of the alkyl side chain of 1 with a basic side chain would be expected to increase secondary binding at anionic phosphate groups in the DNA helix.

Chemistry. Synthesis of the bis(quinaldine) derivatives **2–6** generally involved direct condensation of aliphatic di- or triamines with 4-chloro-2-methylquinolines in phenol.[5,6] The intermediate crotonate **7** (Scheme I), for the preparation of the 4-chloro-2-methylquinoline derivative **10**, was prepared in 76% yield by condensing[5-8] *m*-nitroaniline with ethyl acetoacetate in absolute EtOH with a catalytical amount of glacial acetic acid in the presence of Drierite. Cyclization of **7** in Dowtherm at 250 °C afforded a mixture of 5-nitro- and 7-nitro-2-methyl-4-quinolones **8a,b** in about 50% yield. The separation of the isomers was achieved by treating the mixture with Me_2SO.

Scheme I



The Me_2SO-insoluble solid was shown to be the desired 7-nitro isomer **8b**. The 5-nitro isomer **8a** was recovered from the Me_2SO solution by dilution with H_2O. The structural assigments of these isomers were based on their conversin to known[10] compounds **9a,b** (5-amino- and 7-amino-2-methylquinolines) according to a method similar to one described by Surrey and Hammer[6] and shown in Scheme I. Reaction[11] of **8b** with $POCl_3$ then afforded the 4-chloro compound **10** which upon reaction with the appropriate amines in dry phenol[12] afforded dinitrobis(quinaldines) **11–15** in excellent yields. Reduction[13] of the nitro groups with Fe–$FeCl_3$ in aqueous MeOH gave the desired amino derivatives **2–6**. Many of these bis(quinaldines) (**11–15**, **2–6**) were isolated with solvent of crystallization and gave poor elemental analyses despite repeated purification. Similar purification problems were also encountered by Schock[4] in his preparation of 6-amino derivatives.

Biological Results and Discussion. The antitumor activity (Table I) of the intermediate bis(nitroquinaldines) **11–15** and the target compounds **2–6** was measured in lymphocytic leukemia P-388 by standard protocols[14] of the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health. Compounds are considered active if they give reproducible T/C activity[14] values in the P-388 leukemia system equal to or greater than 125%, where T/C represents the ratio of the mean or median survival times of the treated animals over those of the control animals expressed as a percentage. All T/C results

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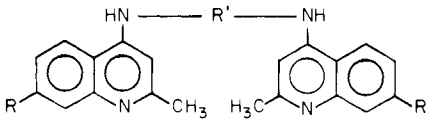
Table I. Physical Properties and Intraperitoneal Lymphocytic Leukemia P-388 Activities[b,c]



Compd	R	R'	Mol formula	Mp, °C dec	R_f[a]	% yield	O.D.[d]	T/C[e]
1	6-NH_2	$(CH_2)_6$	$C_{26}H_{32}N_6 \cdot 2HCl \cdot 2H_2O$				400	254
2	NH_2	$(CH_2)_6$	$C_{26}H_{32}N_6 \cdot 2HCl \cdot 1CH_3OH \cdot 3H_2O$[g]	268-272	0.60	45	25.0	158[f]
3	NH_2	$(CH_2)_7$	$C_{27}H_{34}N_6 \cdot 2HCl \cdot 1CH_3OH \cdot 1H_2O$	260-262	0.63	85	6.25	196[f]
4	NH_2	$(CH_2)_8$	$C_{28}H_{36}N_6 \cdot 2HCl \cdot 2CH_3OH \cdot 1H_2O$	>295	0.70	71	6.25	134[f]
5	NH_2	$(CH_2)_3NH(CH_2)_3$	$C_{26}H_{33}N_7 \cdot 3HCl \cdot 4CH_3OH \cdot 1H_2O$[h]	260-265	0.57	44	12.5	150[f]
6	NH_2	$(CH_2)_4NH(CH_2)_3$	$C_{27}H_{35}N_7 \cdot 3HCl \cdot 2CH_3OH \cdot 4H_2O$[i]	263-266	0.5	62	25	165[f]
11	NO_2	$(CH_2)_6$	$C_{26}H_{28}N_6O_4 \cdot 2HCl \cdot 1CH_3OH$	>295	0.77	99	100	99
12	NO_2	$(CH_2)_7$	$C_{27}H_{30}N_6O_4 \cdot 2HCl$	250-252	0.79	98	12.5	149
13	NO_2	$(CH_2)_8$	$C_{28}H_{32}N_6O_4 \cdot 2HCl \cdot 2CH_3OH$	271-272	0.8	55	12.5	110
14	NO_2	$(CH_2)_3NH(CH_2)_3$	$C_{26}H_{29}N_7O_4 \cdot 2HCl \cdot 2CH_3OH \cdot H_2O$	260-265	0.69	64	25.0	150
15	NO_2	$(CH_2)_4NH(CH_2)_3$	$C_{27}H_{31}N_7O_4 \cdot 2HCl \cdot 4CH_3OH \cdot H_2O$	240-244	0.73	52	12.5	150

[a] *n*-BuOH-AcOH-H_2O (3:1:2) on silica gel (Eastman chromogram 13181). [b] qd 1-9 treatment schedule. [c] H_2O-Tween solution. [d] O.D. = optimum dose, mg/kg. [e] T/C = (treated survival/control) × 100. All T/C results reported for the active compounds have been reproduced in a minimum of one additional experiment to give values not more than 7% lower than the values shown. [f] More H_2O-soluble acetate salts were used. [g] H: calcd, 7.4; found, 6.73. [h] H: calcd, 7.7; found, 6.82. [i] H: calcd, 7.6; found, 6.91.

reported for active compounds have been confirmed in a minimum of one additional experiment.

While the intermediate dinitrobis(quinaldines) **11** and **13** are devoid of antitumor properties (T/C = 99 and 110%, respectively), derivative **12** with 7-methylene groups in the side chain displayed confirmed activity (T/C = 149%) at 12.5 mg/kg. The intermediates **14** and **15**, with an additional imino group in their side chains to enhance secondary binding at anionic phosphate groups in the DNA helix, displayed similar activity (T/C = 150% in each case).

The target compounds **2** and **4** prepared from the corresponding intermediates **11** and **13** are active in this tumor system but do not offer any advantages, including solubility, over the parent compound **1** (T/C = 254%). A comparison between the target compound **3** and the parent **1** reveals significant differences. While the parent compound is very active at 400 mg/kg, **3** is extremely toxic at this dose. At 6.25 mg/kg and lower, **3** displays considerable activity (T/C = 196%), while the parent compound is essentially inactive.

In an effort to gain some insight into the mechanism of action of these drugs, their binding to DNA was examined by (a) elevation of T_m (the temperature at which 50% hyperchromicity is attained owing to heat denaturation of native DNA) and (b) inhibition of DNA function as a template for *E. coli* RNA polymerase in vitro. The T_m studies were carried out according to the method described in the literature.[15-17] The results are presented in Table II.

The results from Table II clearly indicate that these compounds bind to and stabilize the DNA helix toward temperature denaturation. The T_m data also show that the thermal stabilization of DNA does not vary much when the chain length is increased (compounds **1-4**) from 6-methylene units to 8-methylene units, however; it is dependent on the number of basic groups present in the side chains (**5** and **6**). Similar results have been obtained by Burckhalter[17] and are not surprising since polyamines (spermine and spermidine) are known to form complexes with nucleic acids.[18-20]

The dose-dependent inhibition of *Escherichia coli* DNA-dependent RNA polymerase in vitro by these 7-aminobis(quinaldine) derivatives **2-6** is presented in Table III.

Table II. T_m Determination[a]

	T_m, deg	ΔT_m, deg
DNA	68.5	
DNA + 1	86.5	18.0
DNA + 2	87.5	19.0
DNA + 3	85.5	17.0
DNA + 4	87.0	18.5
DNA + 5	91.0	22.5
DNA + 6	88.0	19.5

[a] The drugs were used at a concentration of 1×10^{-5} M in a solution containing 15 μg/mL of calf thymus DNA in 5 mM phosphate buffer at pH 7.4 and the melting temperatures were determined at 260 nm by means of a Gilford 250 recording spectrophotometer with a thermoprogrammer 2527 programmed for a temperature rise of 1.0 °C/min.

Table III. Percent Inhibition of DNA-Dependent RNA Polymerase in Vitro[a]

	Compd				
Concn, M	2	3	4	5	6
1×10^{-4}	97	98	97	94	92
5×10^{-5}	93	98	95	98	94
1×10^{-5}	91	63	95	96	95
7.5×10^{-6}	50	24	91	31	38
5×10^{-6}	29	0	38	9	17
2.5×10^{-6}	0	0	0	0	0

[a] RNA polymerase activity was assayed as described in the Experimental Section. All assays were run in triplicate and did not differ by more than 5%.

The results presented in Table III show that these bis(quinaldine) derivatives **2-6** are strong inhibitors of the RNA polymerase. Strong inhibition of RNA polymerase has also been reported by others[2,17] for diintercalating agents. It is believed[17] that this inhibition results from intercalation of these drug molecules followed by an obstruction of the minor groove, either by the side chain limiting the approach of the enzyme to the minor groove or by the second quinoline ring (which does not intercalate) occupying a trans-like position over the minor groove restricting the approach of the enzyme. Cannellakis et al.,[2] in a study of diacridines as double intercalating agents, have shown that these diacridines are taken up more rapidly by cells than are the monoacridines and appear to

concentrate in the nucleus resulting in a strong inhibition of polymerase at a lower dose (1×10^{-5} M for 50% inhibition for C_8-diacridine). Similar mechanisms, i.e., intercalation followed by obstruction of the minor groove by the side chain and interference by a nonintercalating ring system and/or a rapid transport into the cell nucleus, may be operative for these bis(quinaldine) derivatives **2–6** since these compounds require only $6–9 \times 10^{-6}$ M drug concentration for 50% inhibition of DNA-dependent RNA polymerase reaction.

Experimental Section

Melting points were obtained with a Thomas-Hoover melting point apparatus and are uncorrected. All mass spectra were taken on a LKB 9000 spectrometer with source temperature ~290 °C. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn., and are within ±0.4% of theoretical values, except where indicated.

Ethyl β-(*m*-Nitrophenylamine)crotonate (7). The intermediate β-anilinocrotonic ester **7** was prepared by reacting aromatic amine with ethylacetoacetate according to the method of Hauser and Reynolds.[5] Thus **7** was prepared by reacting a mixture of 55.2 g (0.4 mol) of *m*-nitroaniline and 60.0 g (0.46 mol) of ethyl acetoacetate in 200 mL of absolute EtOH in the presence of 140 g of Drierite and 10 drops of glacial acetic acid. The mixture was refluxed for 18 h, cooled, filtered, and concentrated under reduced pressure. The volatile impurities were removed at 100 °C at 1 mmHg pressure. The residual oil was dissolved in hot $CHCl_3$ and hexane was added dropwise until a turbid solution was obtained. Upon cooling, the yellow solid (starting amine, 5.0 g) was filtered and the mother liquor was concentrated. The residual oil was allowed to crystallize in an ice chest. The yellow solid was triturated with petroleum ether and filtered. Crystallization of the yellow solid with ether–hexane afforded 68.0 g (76%) of pure **7**, mp 55–57 °C. All spectral data (IR, NMR, and MS) are consistent with **7**: *m/e* (rel intensity), 250 (M^+, 28), 233 (8), 205 (16), 204 (14), 189 (12), 187 (9), 177 (12), 164 (11), 163 (100), 161 (13), 159 (14), 131 (18), 130 (40), 117 (35), 103 (8), 92 (8), 91 (9), 90 (7), 89 (8), 77 (9), 76 (32).

7- and 5-Nitro-2-methyl-4(1*H*)-quinolones (8). Cyclization of the crotonate **7** was carried out in Dowtherm at 250 °C. The crotonate (100 g, 0.4 mol) was added portionwise (10 min) through an addition tube to 200 mL of Dowtherm maintained at 250 °C with stirring. The heating was continued for another 30 min and cooled. The mixture was diluted with petroleum ether and the dark solids were collected by filtration. The solids were washed liberally with MeOH and Et_2O.

Separation of Isomers. The resulting mixture of 7- and 5-nitro-2-methyl-4(1*H*)-quinolones (56.0 g) was stirred with 150 mL of Me_2SO for 30 min at room temperature and filtered. The 7-nitro isomer (insoluble solid) **8b** was washed well with H_2O and MeOH and dried. This gave 20.0 g (44%) of **8b** as green solid, mp >300 °C. Mass spectral data are consistent with the structure: *m/e* (rel intensity) 204 (M^+, 100), 174 ($M^+ - NO$, 8), 158 ($M^+ - NO_2$, 56), 146 [$M^+ - (30 + 28)$, 13], 130 ($M^+ - 74$, 20), and 103 (20).

The bulk of the crude 5-nitro isomer **8a** (22.5 g, 56%) was obtained from the Me_2SO filtrate, by dilution with H_2O, as a dark solid, mp >300 °C. The mass spectral data are consistent with the structure: *m/e* (rel intensity) 204 (M^+, 100), 188 ($M^+ - 16$, 7), 174 ($M^+ - NO$, 37), 158 ($M^+ - NO_2$, 47), 146 [$M^+ - (30 + 28)$, 25], 130 ($M^+ - 74$, 57), and 103 (30).

7-Amino-2-methylquinoline (9b). This was prepared from the Me_2SO-insoluble nitro compound **8b** (1.02 g, 0.005 mol) by converting it into the corresponding 4-chloro derivative **10** by treatment with excess $POCl_3$ (4 mL) for 2 h at 125–130 °C. The mixture was cooled, poured in ice–H_2O with stirring, and basified with concentrated NH_4OH. The black solids were filtered and washed well with H_2O. Crystallization from Me_2CO–H_2O afforded 0.8 g (72%) of pure **10** as a light gray solid, mp 167.5–169 °C.

To this 4-chloro derivative **10** (0.666 g, 0.003 mol) suspended in absolute EtOH was added 2 equiv of NaOH, and the mixture was catalytically reduced in the presence of W_2 type[21] Raney nickel. After the theoretical amount of H_2 (for $-NO_2$ and $-Cl$) was absorbed, the catalyst was filtered and the filtrate concentrated under reduced pressure. The residual gummy solid was treated with Et_2O and the Et_2O solution concentrated to an oil. Two crystallizations from CCl_4 afforded pure 7-amino-2-methylquinoline (**9b**) as a pale yellow solid, mp 143–145 °C (lit.[10] 145 °C); the picrate had mp 215–216 °C (lit.[10] 216–217 °C). A strong fluorescence, characteristic[10] only of the 7-amino compounds, was observed on treatment of **9b** with dilute HCl.

5-Amino-2-methylquinoline (9a). This was prepared from the Me_2SO-soluble nitro isomer **8a** similarly by first preparing the 5-nitro-4-chloro-2-methylquinoline in 50% yield, mp 85–87 °C (MeOH–H_2O), followed by catalytic reduction in the presence of W_2 type Raney nickel in absolute EtOH. Work-up and crystallization from $CHCl_3$–petroleum ether gave pure 5-amino-2-methylquinoline (**9a**), mp 110–112 °C (lit.[10] 117–118 °C, anhydrous). No fluorescence was observed on treatment of **9a** with dilute HCl.

***N,N'*-Bis(2-methyl-7-nitro-4-quinolinyl)-1,6-hexanediamine Dihydrochloride (11).** The following reaction is typical for the preparation of bis-nitro derivatives **11–15** listed in Table I, utilizing 7-nitro-4-chloro-2-methylquinoline (**10**). To a solution of 6.66 g (0.03 mol) of **10** in 15 mL of phenol (previously dried at 130 °C for 1 h), 1.74 g (0.015 mol) of 1,6-hexanediamine was added and the mixture heated to 130–135 °C for 18 h. The reaction mixture was cooled to 70 °C and poured into 250 mL of cold acetone with stirring. The crude yellow salts (8.4 g, 99%) were filtered and washed well with Et_2O. Purification of **11** was accomplished by suspending in H_2O, acidifying with dilute HCl, and filtering. The solids were washed well with H_2O and dried. Crystallization from 95% MeOH afforded pure **11**, mp >295 °C. Anal. ($C_{26}H_{28}N_6O_4 \cdot 2HCl \cdot CH_3OH$) C, H, N.

The fragmentation pattern exhibited at 70 eV for **11** is typical for the bis-nitro derivatives **11–13**: *m/e* (rel intensity) 488 (M^+, 12), 471 ($M^+ - 17$, 36), 458 ($M^+ - 30$, 6), 441 (9), 286 (11), 273 (32), 272 (49), 258 (11), 256 (11), 244 (13), 242 (17), 231 (21), 230 (100), 217 (41), 216 (47), 203 (20), 200 (30), 172 (32), 169 (59).

***N,N'*-Bis(2-methyl-7-amino-4-quinolinyl)-1,6-hexanediamine Diacetate (2).** The following reaction is typical for preparation of the 7-amino derivatives **2–6** from the corresponding dinitro compounds **11–15**. The dinitro compound **11** (5.0 g, 0.008 mol) was suspended in 80% MeOH and heated to reflux. When all the dinitro compound had dissolved, 2.5 g of Fe powder (150 g/mol of nitro group) and 1.5 mL of 32.5% $FeCl_3$ solution (20 mL/mol of nitro group) were added. The heating was continued until black Fe_3O_4 formed (6–8 h), concentrated NH_4OH was added, and the mixture was filtered hot. The solids were washed with hot MeOH. The combined filtrate was evaporated to dryness under reduced pressure, the dark residue was dissolved in dry MeOH, and excess glacial AcOH was added. After stirring for 2 h at room temperature the mixture was filtered and the MeOH solution was added dropwise to Et_2O with stirring. The red-brown solids were filtered, washed with a small amount of H_2O, and dried. Three crystallizations from MeOH–Et_2O afforded 2.2 g (45%) of **2** as red-brown solid, mp 270–272 °C dec. The dihydrochloride salts (H_2O–HCl, 95% MeOH–Me_2CO) had mp 268–272 °C dec. Anal. ($C_{26}H_{32}N_6 \cdot 2HCl \cdot CH_3OH \cdot 3H_2O$) C, N, Cl; H: calcd, 7.49; found, 6.73.

The fragmentation pattern exhibited at 70 eV for **2** is typical for all these derivatives (**2–6**): *m/e* (rel intensity) 428 (M^+, 29), 256 (29), 242 (70), 228 (11), 214 (23), 200 (100), 187 (58), 186 (70), 174 (29), 173 (38).

Biological Testing. RNA Polymerase. Calf thymus DNA, *E. coli* RNA polymerase, and the nucleoside triphosphates were purchased from Sigma Chemical Co. The [8-^{14}C]-ATP (50 mCi/mM) was obtained from New England Nuclear. The activity of RNA polymerase was determined by measuring the amount of [^{14}C]-ATP rendered acid insoluble as determined by the filter paper disk assay of Bollum.[22] The incubation mixture contained in 0.125 mL: Tris–HCl buffer, pH 8.0, 5 μmol; $MgCl_2$, 1.25 μmol; β-mercaptoethanol, 1.25 μmol; CTP, UTP, and GTP each 0.019 μmol; [8-^{14}C]-ATP, 0.019 μmol (65 000 cpm); calf thymus DNA, 3.65 μg. The compounds to be tested were dissolved in MeOH and 5 μL of the solution was added to the incubation mixture just prior to the addition of the enzyme. Controls contained 5 μL of MeOH. The reaction mixtures were incubated for 10 min at 37 °C. The acid-insoluble material from an aliquot (100 μL) of each incubation mixture was isolated by the procedure of Bollum,[22]

placed in 18 mL of scintillation fluid [6.0 g of 2,5-diphenyloxazole, 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 1400 mL of toluene, and 600 mL of MeOH], and counted in a Beckman LS-230 liquid scintillation spectrometer. In the absence of added drug, 1.05 mmol of [8-^{14}C]-ATP was incorporated into DNA during the 10-min incubation period.

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Diastereoisomers of 5,10-Methylene-5,6,7,8-tetrahydropteroyl-D-glutamic Acid

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The diastereoisomers of 5,10-methylene 5,6,7,8-tetrahydropteroyl-D-glutamate were resolved and tested as substrates and inhibitors of *Lactobacillus casei* thymidylate synthetase. No activity was observed. The compounds were neither growth factors nor inhibitors for *Lactobacillus casei*, *Streptococcus faecium*, or *Pediococcus cerevisiae*. 7,8-Dihydropteroyl-D-glutamate is 50% as active as 7,8-dihydropteroyl-L-glutamate (dihydrofolate) as a substrate for *L. casei* dihydrofolate reductase.

Studies with diastereoisomers of 5,6,7,8-tetrahydropteroyl-L-glutamate and derivatives (tetrahydrofolates) at carbon 6 (Figure 1) have shown that in some biological systems one configuration, *l*, is active whereas the other, *d*, is inert. (Since the absolute configurations at carbon 6 are not established, the configuration found in naturally occurring tetrahydrofolate derivatives is arbitrarily designated *l* and its enantiomer *d*.) Examples include the growth of *Pediococcus cerevisiae* on 5-formyl 5,6,7,8-tetrahydropteroyl-L-glutamate[1] and the reaction catalyzed by chicken liver 5,10-methenyltetrahydrofolate cyclohydrolase.[2] However, *d*-5,10-methylene-5,6,7,8-tetrahydropteroyl-L-glutamate inhibits *Escherichia coli* methylenetetrahydrofolate dehydrogenase[3] and *Lactobacillus casei* thymidylate synthetase.[4] Furthermore *d*-5,6,7,8-tetrahydrohomopteroyl-L-glutamate is a potent inhibitor of the growth of *L. casei*[5] and *Streptococcus faecium*,[6] the corresponding *l* form being a growth factor. In view of these varied effects of stereochemical configuration on biological activity, we prepared and tested the diastereoisomers of 5,10-methylene 5,6,7,8-tetrahydropteroyl-D-glutamate.

Results and Discussion

The pattern of elution of the diastereoisomers of methylene tetrahydropteroyl-D-glutamate from diethylaminoethyl cellulose columns corresponds to that observed for the corresponding L-glutamate derivatives.[7,8] The peak emerging from the column first could be assigned the *d* configuration at carbon 6 because it has a negative ellipticity at 285 nm (Figure 2) as does *d*-5,10-methylene tetrahydropteroyl-L-glutamate.[4] The ellipticity contributed by the D-glutamate residue at 285 nm is 20% that caused by the asymmetric center at carbon 6 and therefore does not interfere with this assignment. (The ellipticity values reported[4] for methylenetetrahydrofolate diastereoisomers were inadvertently 0.1 of the correct values[9]). The second peak emerging from the column is assigned the *l* configuration at carbon 6 because its ellipticity (Figure 2) corresponds to *l*-5,10-methylene tetrahydropteroyl-L-glutamate.[4] The ultraviolet absorption spectrum of both diastereoisomers was the same, consisting of a single peak at 295 nm expected for 5,10-methylenetetrahydrofolate.[7]

Enzymatic Tests. Neither diastereoisomer showed cofactor activity for *L. casei* thymidylate synthetase whether the enzyme was assayed spectrophotometrically[10] or by the release of ^{3}H from deoxyuridine 5′-phosphate [5-^{3}H][11] (Table I). The K_m for *l*-5,10-methylene tetrahydropteroyl-L-glutamate under the same conditions is 1.5×10^{-5} M.[4]

Neither diastereoisomer inhibited thymidylate synthetase (Table I). For comparative purposes *d*-5,10-methylene tetrahydropteroyl-L-glutamate is shown to produce detectable inhibition under similar conditions