8-Methoxypsoralen–Nucleic Acid Photoreaction. Effect of Methyl Substitution on Pyrone vs. Furan Photoaddition

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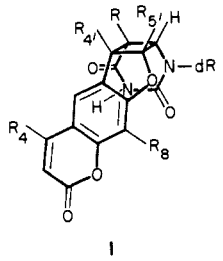
We have synthesized a series of 8-[^{3}H]methoxypsoralens in which methyl and hydrogen are systematically varied at the 4- and 5′-positions. Analysis of the products resulting from the photoaddition of these four psoralens with the nucleic acid poly(dA-dT) reveals that the product distribution depends on the presence or absence of a 4-methyl substituent. Compounds with the 4-methyl group show an overwhelming preference (~98%) for addition to the furan double bond, while compounds without the 4-methyl show a substantial amount (~18%) of addition to the pyrone double bond.

The linear furocoumarins, commonly known as psoralens, have seen wide use as dermal photosensitizing agents[1,2] and as probes of nucleic acid strucutre and function.[3] This biological activity of psoralens is primarily the result of the covalent bonding they undergo with nucleic acids. The process is believed to involve three major steps: (1) noncovalent intercalative binding to the DNA helix, (2) formation of a monoaddition product between the psoralen and a DNA base upon long-wavelength ultraviolet irradiation, and (3) absorption of a second photon by some of the monoadducts to form diadducts, resulting in interstrand cross-links. It has recently been shown that of the two types of monoadducts, furan side and pyrone side, only the former is capable of forming a diadduct at 365-nm irradiation.[4] An understanding of the effect of peripheral substituents on the relative amounts of photoaddition at the competing furan and pyrone double bonds would therefore be invaluable in designing psoralens in which the ability to perform a specific function, such as the ability to cross-link or to form only monoadducts, is maximized.

The isolation and complete structural characterization of the major nucleoside–psoralen monoadducts (1 and 2)



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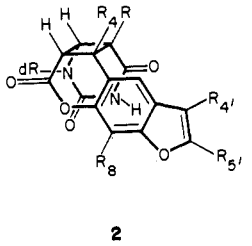

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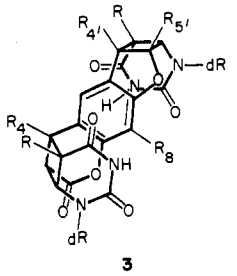

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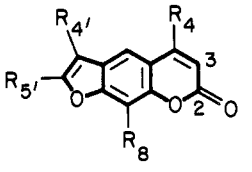
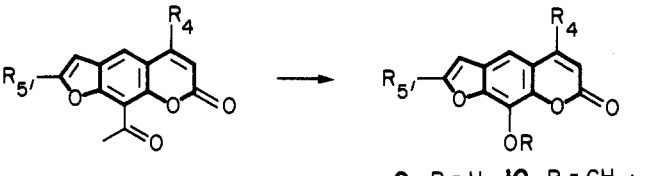
and nucleoside–psoralen–nucleoside diadducts (3) of a number of important psoralens (4–7) have recently been



4, $R_4 = R_{4'} = R_{5'} = R_8 = H$
5, $R_4 = R_{5'} = R_8 = CH_3$, $R_{4'} = H$
6, $R_4 = R_{4'} = R_{5'} = H$, $R_8 = OCH_3$
7, $R_4 = R_{5'} = R_8 = CH_3$, $R_{4'} = CH_2OH$

reported.[5,6] The methodology described in those reports allowed for the quantification of the mono- and diadducts formed in a given psoralen–nucleic acid reaction. Only tentative conclusions, however, could be reached concerning the effect of the peripheral substituents on addition at the two photoreactive double bonds, since there was no systematic variation of the substitution pattern in the psoralens studied.

In the present study we have synthesized a series of 8-[^{3}H]methoxypsoralens in which CH_3 and H are systematically varied at the 4- and 5′-positions, shown in **10a–d**.



8 **9**, R = H; **10**, R = CH_3; **11**, R = C^3H_3

a: $R_4 = R_{5'} = H$
b: $R_4 = CH_3$, $R_{5'} = H$
c: $R_4 = H$, $R_{5'} = CH_3$
d: $R_4 = R_{5'} = CH_3$

Analysis of the photoproducts from the reaction of these four psoralens with the nucleic acid poly(dA-dT)·poly(dA-dT) has shown that the 4-CH_3 group is the controlling factor in the product distribution observed in this series. Compounds with a 4-CH_3 show an overwhelming preference for furan addition (~98%), while compounds without the 4-CH_3 show a substantial amount of pyrone addition (~18%).

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Results

The psoralen-modified nucleic acids[7] were hydrolyzed enzymatically, and the resulting hydrolysis mixture contained the psoralen-modified nucleosides along with a much larger quantity of material due to the unmodified nucleosides. Isolation of the individual adducts from this complex sample matrix was accomplished by high-performance liquid chromatography (HPLC).

The results of earlier NMR studies[4-6] on 8-methoxypsoralen (8-MOP) and three other psoralens have firmly established the generality of the cis–syn stereochemistry of the various adducts **1–3**. Differentiation of the various adducts [furan monoadducts (**1**) and pyrone monoadducts (**2**), and diadducts (**3**)] was achieved by their absorption spectra and photoreversion behavior. The absorption spectra of the furan monoadducts have a characteristic maximum at about 326 nm. This absorption is typical of the coumarin chromophore of a 4′,5′-dihydropsoralen. Photoreversion (irradiation at 254 nm) of the furan monoadducts yields the parent psoralen and thymidine as the only two products. Neither the pyrone monoadduct nor the diadduct has an absorption maximum above 300 nm. Photoreversion experiments provided a reliable method for differentiating between these two adduct classes. The diadduct[8] reverts to a mixture of pyrone monoadduct, furan monoadduct,[9] the parent psoralen, and thymidine, while the monoadduct reverts simply to thymidine and the parent psoralen.

In all cases studied, the peaks eluting between 5 and 20 min do not contain ^3H and are due to the unmodified nucleosides. The major ^3H-containing peaks in the case of 8-MOP (**10a**) modified poly(dA-dT)·poly(dA-dT) are fractions F32, F38, and F41. These compounds are identical with those already isolated and fully characterized[4-6] from DNA: F32 is the thymidine–8-MOP–thymidine diadduct; F38 is the thymidine–8-MOP furan monoadduct,[10] and F41 is the thymidine–8-MOP pyrone monoadduct. The amount of addition at the pyrone side was about 19%.[11]

The major ^3H-containing peaks for 4,5′-Me_2-8-MOP (**10d**) are F34 and F35 (diadducts), F45A and F45B (furan monoadducts), and F48 (pyrone monoadducts). The diadduct F36 undergoes conversion to F34 at pH 2.2. This pH-dependent transformation that occurs with diadducts and pyrone monoadducts has been noted before[4] and is due to hydrolysis of the saturated lactone to a δ-hydroxy acid. At acid pH the lactone species F34 is favored. Photoreversion of either F34 or F36 gives a mixture of furan and pyrone monoadducts.

The diastereomeric furan monoadducts F45A and F45B give virtually identical absorption spectra with a λ_{max} at 326 nm and photorevert to thymidine and the parent psoralen. The pyrone-monoadduct F48 has no absorption above 300 nm, gives the expected photoreversion products, and represents only 2% of the total adducts formed. Thus, the overwhelming preference for addition at the furan side for this derivative is similar to that of 4,5′,8-trimethylpsoralen.[6]

Table I. Product Distribution (Furan Monoadduct plus Diadduct vs. Pyrone Monoadduct) after 10-min Irradiation at 360 nm

			radioact., %		
no.	R_4	$R_{5'}$	pyrone monoadduct	furan monoadduct + diadduct[a]	total radioact. accounted for, %
10a	H	H	19	75	94
10b	CH_3	H	2	94	96
10c	H	CH_3	17	73	90
10d	CH_3	CH_3	2	94	96

[a] See ref 11.

4-Me-8-MOP (**10b**) showed major ^3H-containing fractions at F32 and F34 (diadducts), F42 and F43 (furan monoadducts), and F45 and F47 (pyrone monoadducts). The diadducts again exhibit a pH-dependent interconversion between hydroxy acid (F34) and lactone (F32). The diastereomeric furan monoadducts are F42 and F43 with λ_{max} at 326 nm. Again, the pyrone-side monoadducts F45 and F47 show an interconversion between hydroxy acid and lactone similar to that observed for the diadducts. Opened lactone F47 is the predominant species obtained after enzymatic hydrolysis at pH 8.0 and 37 °C. There also is present a smaller quantity (~20% of total pyrone fraction) of F45 (intact lactone), and hydroxy acid F47 is converted slowly to lactone F45 at pH 2.2 and room temperature. The pyrone monoadducts account for only 2% of the total adducts.

The major adducts for 5′-Me-8-MOP (**10c**) appear at F35 (diadduct), F43 (furan monoadduct), and F45 (pyrone monoadduct). The pyrone monoadduct accounted for 17% of the total adducts.

Conclusions

The relative amounts of photoaddition at the furan and pyrone side for the four derivatives studied is summarized in Table I, from which it is clear that the predominant reaction for all four derivatives studied is at the furan side. This preference is especially pronounced for the two derivatives containing a methyl group at C-4.

A pulse–chase study[4] has shown that continued irradiation of 8-MOP-modified poly(dA-dT) produces a mixture that contains only cross-link (generated exclusively from furan monoadduct) and pyrone monoadduct (a dead-end product). A knowledge of the amount of pyrone monoadduct provides a minimum value for the amount of adduct that cannot be converted to cross-link. Therefore 8-MOP and 5′-Me-8-MOP will ultimately produce less cross-link in poly(dA-dT)[12] than will 4-Me-8-MOP and 4,5′-Me_2-8-MOP because of the enhanced yield of pyrone monoadduct with the former compounds.

The pyrimidine–psoralen photoadducts (**1–3**) isolated from nucleic acids have been shown to possess cis–syn stereochemistry.[4-6] This stereochemistry is a direct consequence of an intercalation complex in which there is maximum overlap between the psoralen and the base pairs

(7) The covalent photoaddition in terms of number of nucleic acid base pairs per covalently bound psoralen for the various derivatives was as follows: **10a**, 31; **10b**, 8; **10c**, 200; **10d**, 16.

(8) On reverse-phase HPLC the diadduct, carrying two dihydropyrimidine moieties, had a retention time at least 8 min shorter than the pyrone-side monoadduct.

(9) Cleavage of the furan-side cyclobutane proceeded at a faster rate than the pyrone-side cyclobutane.

(10) The diastereomer of F38 (F37), which was isolated in significant quantities (50% of F38) in the case of DNA, is isolated in only small amounts (5% of F38) in the case of poly(dA-dT).

(11) It has been shown that the diadduct is formed by photocycloaddition of a second thymidine residue to the 3,4 double bond (pyrone side) of an initially formed 4,5′ (furan-side) monoadduct (see ref 4).

(12) While every cis–syn furan monoadduct in poly(dA-dT) has a thymidine positioned in the appropriate position on the opposite strand for cross-link formation, this is not the case in other nucleic acids. The amount of cross-link ultimately produced in other nucleic acids will therefore be limited not only by the amount of pyrone monoadduct produced but also by the specificity of a given psoralen for forming furan monoadducts at cross-linkable sites.

of the nucleic acid. Alternative product stereochemistries would derive from intercalation complexes where the psoralen is protuding from the helix. Examination of models of the intercalation complex that gives rise to the observed cis–syn pyrone monoadduct reveals that the C-5 methyl of thymidine and the C-4 substituent of the psoralen are in close proximity. Thus, the presence of a methyl in this position could lead to steric crowding not present in the demethyl case. This explanation is consistent with the results summarized in Table I where the presence of the 4-methyl group lowers the amount of pyrone monoadduct to 2% or less.[13] The results obtained previously[3,4] for TMP (**5**), HMT (**7**), and psoralen (**4**) lend further support to this interpretation. TMP and HMT, both with a 4-methyl group, showed ~98% furan addition, while psoralen, lacking a methyl at the 4-position, showed ~20% pyrone addition.

The results of this study on the photoreaction of a series of psoralens with the nucleic acid poly(dA-dT)·poly(dA-dT) have revealed the effects of olefinic methyl substituents on the relative amounts of photoaddition at the furan and pyrone double bonds. Further structure–activity relationships encompassing both electronic and steric variations of peripheral substituents are currently under investigation.

Experimental Section

5′-Methyl-8-hydroxypsoralen (9c). A suspension of 40 mg (0.165 mmol) of the acetyl compound **8c**[14] in 10 mL of 2 N NaOH was heated for 15 min and then cooled in an ice bath to 0 °C, and H_2O_2 was added dropwise with stirring until the reaction was complete (TLC). The reaction was quenched at 0 °C by the addition of 2 N HCl to pH 1, and the aqueous mixture was extracted three times with $CHCl_3$/2-propanol (5:1). The combined extracts wre dried over sodium sulfate and evaporated to yield 25 mg (72%) of crude **9c**, purified by prep TLC (silica) using CH_2Cl_2/MeOH: yield 22 mg (61%); mp >240 °C; NMR ($CDCl_3$, CD_3OD) δ 7.78 (d, J = 9.5 Hz, H-4), 7.02 (s, H-5), 6.34 (m, H-4′), 6.25 (d, J = 9.5 Hz, H-3), 2.49 (d, C-4 CH_3). Anal. ($C_{12}H_8O_4$) C, H.

4-Methyl-8-hydroxypsoralen (9b) and 4,5′-dimethyl-8-hydroxypsoralen (9d) were prepared in the same manner as described above. **9b**: mp >240 °C; NMR ($CDCl_3$) δ 7.70 (d, J = 2.2 Hz, H-5′), 7.38 (s, H-5), 6.82 (d, J = 2.2 Hz, H-4′), 6.25 (m, H-3), 2.49 (d, C-4 CH_3). Anal. ($C_{12}H_8O_4$) C, H. **9d**: mp >240 °C; NMR ($CDCl_3$) δ 7.25 (s, H-5), 6.41 (m, H-4), 6.24 (m, H-3), 2.51 (d, H-5), 2.49 (d, H-3). Anal. ($C_{13}H_8O_4$) C, H.

8-Hydroxypsoralen (9a) was prepared from 8-methoxypsoralen by treatment with BBr_3 as described previously,[15] mp 247–249 °C (lit.[15] mp 247–249 °C).

5′-Methyl-8-methoxypsoralen (10c). A suspension of 60 mg (0.28 mmol) of **9c**, 76.6 mg (0.55 mmol) of K_2CO_3, 1 mL of acetone, and 43.2 mg (0.30 mmol) of methyl iodide was heated at reflux for 6 h, then the acetone was evaporated, and the residue was partitioned between $CHCl_3$ and water. The water layer was extracted with $CHCl_3$ (3 × 50 mL), and the combined organic phases were dried with sodium sulfate and evaporated to yield 56 mg (88%) of **10c**: mp 151–152 °C (lit.[16] mp 151–152 °C); NMR ($CDCl_3$) δ 7.7 (d, J = 9.5 Hz, H-4), 7.2 (s, H-5), 6.4 (m, H-4′), 6.35 (d, H-3), 4.3 (s, OCH_3), 2.5 (d, C-5′ CH_3). Calcd for $C_{13}H_{10}O_4$: 230.0579. Found: m/z 230.0579 (M^+).

Compounds **10b**, **10d**, and **10a**[15] were prepared in the same manner as described for **10c**.

4-Methyl-8-methoxypsoralen (10b): NMR ($CDCl_3$) δ 7.7 (d, 2.2 Hz, H-5′), 7.5 (s, H-5), 6.8 (d, H-4′), 6.3 (m, H-3), 4.3 (s, OCH_3), 2.5 (d, C-4 CH_3); mp 167–168 °C (lit.[16] mp 167–168 °C). Calcd. for $C_{13}H_{10}O_4$: 230.0579. Found: m/z 230.0574 (M^+).

4,5′-Dimethyl-8-methoxypsoralen (10d): NMR ($CDCl_3$) δ 7.35 (d, H-5), 6.4 (m, H-4′), 6.24 (m, H-3), 4.26 (s, 8-OCH_3), 2.5 (d, C-5′ CH_3), 2.47 (d, C-4 CH_3); mp 158–159 °C (lit.[16] mp 158–159 °C). Calcd for $C_{14}H_{12}O_4$: 244.0735. Found: m/z 244.0730 (M^+).

Synthesis of Radiolabeled Derivatives. 4,5-Dimethyl-8-[$^3H_3$]methoxypsoralen (11d). A 10-mL pear-bottom flask to which a glass stopcock had been attached served as a low-pressure autoclave for the reaction. 4,5-Dimethyl-8-methoxypsoralen (**10d**; 23 mg, 0.10 mmol), potassium carbonate (27.6 mg, 0.20 mmol), and acetone (1.0 mL) were added to the reaction flask, followed by [$^3H_3$]methyl iodide (50 mCi, 2–3 Ci/mmol) in acetone (0.5 mL). The valve was shut, and the mixture heated at 50 °C for 15 h with magnetic stirring, after which the solution was frozen, and the solvent was removed under high vacuum. The residue was dissolved in water (5 mL) and extracted with chloroform (4 × 3 mL), and the chloroform was washed with water, dried over sodium sulfate, and evaporated. Initial fractionation of the residue on a prep TLC plate (silica) with dichloromethane was followed by HPLC analysis, and the fraction collected was found to be greater than 98% radiochemically pure. The yield was about 50% based on the amount of [$^3H_3$]methyl iodide used. The specific activity of the derivatives used in the photoreactions was about 150 000 dpm/μg. The other radiolabeled derivatives, **11a–c**, were synthesized in the same manner.

Photobinding. To 1 mg of alternating poly(dA-dT)·poly(dA-dT) (1.5 μmol of base pairs) in 1 mL of Tris buffer (10 mM, pH 7.2) was added 0.3 μmol of the tritiated psoralen dissolved in 95% EtOH. This solution was irradiated for 10 min at 10 °C. Two 400-W GE mercury vapor lamps were used for the irradiation, and cooling of the solution was achieved by circulation of a solution of cobaltous nitrate (40%, w/w) through an outer jacket; this solution also served as a 365-nm transmission filter. The intensity of the light at the surface of the inner sample chamber in this device is approximately 100 mW/cm^2. After the 10-min irradiation, the psoralen–DNA solution was extracted with 4 vol of chloroform to remove unreacted psoralen and its photodegradation products. The remaining solution was made 0.2 M in sodium chloride, diluted with 3 vol of cold ethanol, and allowed to stand in a dry ice–acetone bath for 1 h. The precipitate of polymer was collected by centrifugation, dissolved in 0.2 M sodium chloride, and reprecipitated by addition of ethanol. The isolated nucleic acid pellet was then dried under vacuum and redissolved in enough hydrolysis buffer (15 mM sodium acetate , pH 5.00) to give a concentration of 2 mg/mL.

Adduct Isolation. The psoralen-modified nucleic acid (ca. 1 mg/mL in 15 mM NaOAc, pH 5) was first hydrolyzed at 37 °C by the addition of 80 units of DNase II (EC 3.1.22.1) per milligram of nucleic acid. After 24 h, the solution was adjusted to pH 7 with Tris base, and 0.2 unit of phosphodiesterase II (EC 3.1.4.18) per milligram of nucleic acid was added. After 24 h, a second addition of phosphodiesterase was made, followed by another 24 h of hydrolysis, at which time the pH was adjusted to 7.8–8.0 and 0.2 unit of alkaline phosphatase (EC 3.1.3.1) per milligram of nucleic acid was added. After 24 h of hydrolysis by the alkaline phosphatase, the pH was adjusted to 3, and the mixture was centrifuged to produce a clear supernatant. The mixture was then analyzed by reverse-phase HPLC on an Ultrasphere ODS column (either 4.6 or 10 mm × 25 cm; Altex). Water–methanol (0–10 min, 0% MeOH; 10–70 min, linear gradient to 100% MeOH) was used as the eluting solvent at a flow rate of 1 or 4 mL min^{-1}. A buffered aqueous phase was used (10 mM KH_2PO_4, pH 2.2). The column effluent was monitored for absorbance at 254 nm and assayed for the presence of ^3H by scintillation counting.

(13) These same considerations would lead to the prediction that the 4′-hydroxymethyl of 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen should cause a significant decrease in the amount of furan-side monoadduct compared to 4,5′,8-trimethylpsoralen. Such an effect is not observed experimentally (see ref 4 and 5). In this case the potential for a hydrogen-bonding interaction between the 4′-hydroxymethyl of the psoralen and the C-4 carbonyl of the pyrimidine may serve to counterbalance the steric effect. An analysis of the photoadduct ratios of 4,4′,8-trimethylpsoralen would resolve this question.

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Photoreversion. The adduct of interest was dissolved in 50% methanol/water at a concentration of 10 μg/mL. This solution was irradiated at 254 nm with a low-intensity mercury hand lamp for 1–5 min. The solution was then analyzed by HPLC on the Ultrasphere ODS column. Identification of products was achieved by coinjection with authentic standards.

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Registry No. **8b**, 86290-69-9; **8c**, 86290-65-5; **8d**, 14256-46-3; **9a**, 2009-24-7; **9b**, 88589-76-8; **9c**, 88589-77-9; **9d**, 88589-78-0; **10a**, 298-81-7; **10b**, 5263-08-1; **10c**, 5307-52-8; **10d**, 5307-51-7; **11a**, 82460-28-4; **11b**, 88589-79-1; **11c**, 88589-80-4; **11d**, 88589-81-5; poly(dA-dT)·poly(dA-dT), 26966-61-0.

(±)-3-(4-Amino-1*H*-pyrrolo[2,3-*d*]pyrimidin-1-yl)-5-(hydroxymethyl)-(1α,2α,3β,5β)-1,2-cyclopentanediol, the Carbocyclic Analogue of Tubercidin

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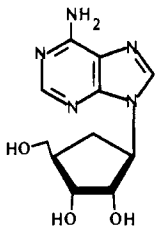
(±)-3-(4-Amino-1*H*-pyrrolo[2,3-*d*]pyrimidin-1-yl)-5-(hydroxymethyl)-(1α,2α,3β,5β)-1,2-cyclopentanediol (**9**), the carbocyclic analogue of tubercidin, prepared from (±)-3-amino-5-(hydroxymethyl)-(1α,2α,3β,5β)-1,2-cyclopentanediol (**6**), is cytotoxic to cells containing adenosine kinase but not to cells that do not, indicating that its activity depends on phosphorylation. Although inactive against P388 leukemia in mice and against herpes and influenza viruses in vitro, it showed marginal activity against respiratory syncytial, vesicular stomatitis, and rhino viruses in vitro.

Carbocyclic analogues of nucleosides have proven to possess interesting biological activities.[1] Carbocyclic adenosine (CAdo, **1**)[2] is cytotoxic to both H.Ep.-2 and



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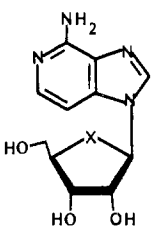
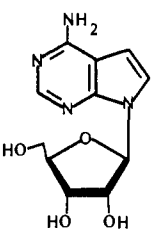


2: X = O
3: X = CH_2



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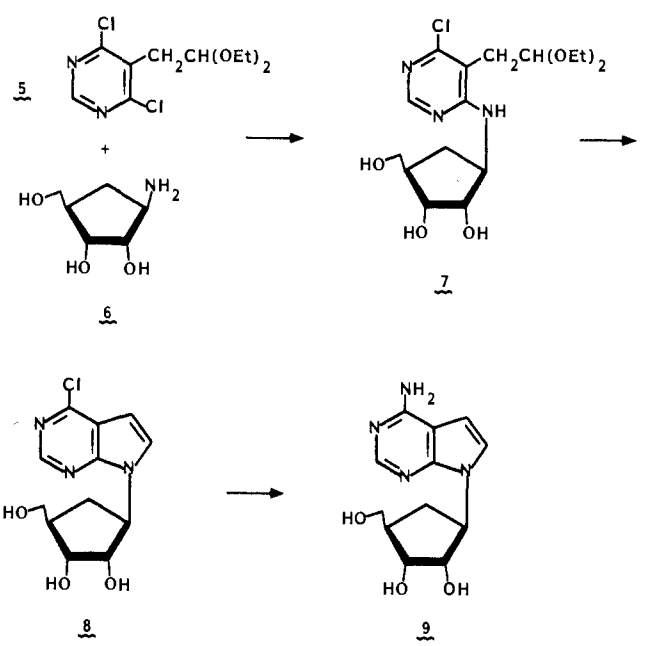
L-1210 cells in culture, although it is not effective against L-1210 leukemia in vivo at the maximum tolerated dose, indicating a lack of selectivity for neoplastic cells. It was inactive against herpes simplex type 1, vaccinia, rhino type 1A, and influenza (Ao/PR-8/34) viruses. CAdo is deaminated by adenosine deaminase and phosphorylated by adenosine kinase.[3] Even so it is cytotoxic to cells deficient in adenosine kinase, indicating that the nucleoside analogue itself can kill cells.[3] CAdo is an extremely potent inhibitor of *S*-adenosyl-L-homocysteinase,[4] the inhibition of which causes a perturbation in biological methylations by effecting a buildup of *S*-adenosyl-L-homocysteine, which is a potent feedback inhibitor of methyltransferases utilizing *S*-adenosylmethionine.[5] These inhibitions may explain the toxicity of CAdo itself to cells that cannot phosphorylate it. 3-Deazaadenosine (3-deaza-Ado, **2**),[6] which is not detectably deaminated[7] and only very poorly

Scheme I



phosphorylated,[8] is also an excellent competitive inhibitor of *S*-adenosyl-L-homocysteinase (although less potent than CAdo)[4] and exhibits some antiviral activity.[9] Carbocyclic 3-deazaadenosine (3-deaza-CAdo, **3**), which is neither phosphorylated nor deaminated, is an equally good inhibitor of *S*-adenosyl-L-homocysteinase[10] and exhibits potent activity against vaccinia and a number of RNA viruses in vitro and activity against VSV and vaccinia in vivo.[9] The activity of 3-deaza-CAdo (**3**) and of tubercidin (7-deazaadenosine, **4**)[11] suggested the synthesis and evaluation of carbocyclic tubercidin [7-deaza-CAdo, (±)-3-(4-amino-1*H*-pyrrolo[2,3-*d*]pyrimidin-1-yl)-5-(hydroxy-

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