which the hydroxy-bearing side-chain carbon is fully substituted, were unacceptable as substrates for NMT; since the compounds in this study are also fully substituted at the equivalent side-chain carbon, this may account for the weak activities found in this study. However, recent results from our laboratory[5] also suggest an alternative explanation that is more directly related to the hydrophobic nature of the compounds in Table I. Evidence suggests that more hydrophobic, nonaromatic analogues of phenylethanolamine are displaced deeper into the hydrophobic ring binding region than are aromatic substrates, such that the side-chain hydroxy loses contact with its corresponding binding site. Since the hydroxy group interaction is known to be important for substrate activity with NMT,[10] this loss of binding interaction results in a loss in substrate activity and also in stereoselectivity for the substrate. It is therefore conceivable that compounds 1–3 are also bound so deep within the hydrophobic binding region that the side-chain hydroxy is unable to contact its binding site; thus, methylation of these compounds cannot occur to any significant extent.

In summary, we have found that the *trans*-decalin analogues 2 and 3 of a known alicyclic inhibitor (1) of NMT demonstrated equivalent inhibitory potency. These results suggest that the hydrophobic region of the ring binding site of NMT is a relatively flat area, which best accommodates structures having an overall planar shape or which are able to assume such a shape. The amino group binding site is indicated by the activity of these compounds to lie slightly out of the plane of the hydrophobic region, in an area which is accessed by two rather distinct side-chain orientations. These results will be applied to further investigations of the NMT active site.

Experimental Section

Melting points were taken on a Thomas-Hoover capillary melting point apparatus calibrated with known compounds. NMR spectra were recorded on a Varian A-60A spectrometer in trifluoroacetic acid, with Me_4Si as the internal reference. Combustion analyses were performed on a Hewlett-Packard 185B CHN Analyzer. IR spectra were recorded on a Beckman IR 33 spectrophotometer. β-Decalone was purchased from Aldrich Chemical Co., and cyanotrimethylsilane was obtained from Petrarch Chemical Co. The axial isomer 3 was generously provided to us by Dr. R. G. Carlson, University of Kansas.

2(*e*)-(Aminomethyl)-*trans*-2-decalol (2). The method of Evans et al.[11] was used. Into a flame-dried, N_2-purged flask was placed 3.27 g of Me_3SiCN (33 mmol) and 60 mg of ZnI_2 catalyst. The mixture was cooled to 0 °C, and 5.0 g of β-decalone (33 mmol) was added dropwise via syringe with stirring. The mixture was allowed to warm slowly to room temperature, and the progress of the reaction was monitored by IR spectroscopy for residual C=O stretching frequency at 1712 cm^{-1}. Complete disappearance of this band occurred within 30 min; a weak nitrile band was detected (2230 cm^{-1}). The pot contents were drawn into a syringe and added dropwise to a slurry of 3.0 g of $LiAlH_4$ in dry ether at a rate of addition that maintained a gentle reflux. After the addition was complete, the reaction was allowed to stir at room temperature for 16 h. After quenching the excess $LiAlH_4$ with 3 mL of H_2O, followed by 5 mL of 1 N NaOH, the reaction was filtered and the filtrate was extracted with ether (3 × 25 mL). The pooled ether layers were dried (K_2CO_3) and evaporated, leaving a white residue, which was recrystallized from ether to yield 4.45 g (72%) of white needles, mp 90–93 °C. NMR analysis (trifluoroacetic acid) showed the appearance of a quartet at 3.49 ppm, which was assigned to the side-chain methylene. It was reported that the chemical shift of this methylene of the equatorial isomer (2) was 3.53 ppm; the signal for the axial isomer appeared at 3.29 ppm.[6] No signals could be detected downfield from 3.49 ppm, confirming the absence of any of the axial isomer 3. Further purification of the product was achieved by sublimation (75 °C, 0.1 mm), to yield a white solid, mp 90 °C (lit.[6] mp 92 °C). Anal. ($C_{11}H_{21}NO$) C, H, N.

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Mechanism of Action of 5-Nitro-2′-deoxyuridine

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Results are described that demonsrate that the mechansim of action of the potent cytotoxic agent 5-nitro-2′-deoxyuridine (NO_2dUrd) involves thymidine (dThd) kinase catalyzed formation of 5-nitro-2′-deoxyuridylate (NO_2dUMP) and subsequent potent inhibition of thymidylate (dTMP) synthetase by this compound. The evidence for this is as follows: (a) cells lacking dThd kinase are not inhibited by high concentrations of NO_2dUrd; (b) the drug has no effect on dThd or uridine (Urd) incorporation into nucleic acids but prevents incorporation of deoxyuridine (dUrd); (c) growth inhibition is reversed by dThd but not by dUrd; (d) NO_2dUrd causes changes in deoxynucleoside triphosphate pool sizes which are characteristic of specific inhibition of dTMP synthetase; (e) cells treated with [^{3}H]NO_2dUrd possess macromolecular bound [^{3}H]NO_2dUMP, which has properties characteristic of the NO_2dUMP–dTMP synthetase complex. Treatment of L1210 leukemic mice at 400 mg/kg daily for 6 days gave only a 33% increase in life span, probably because of its rapid degradation to the inactive nitrouracil.

Thymidylate synthetase (EC 2.1.1.45) catalyzes the conversion of 2′-deoxyuridylate (dUMP) and methylenetetrahydrofolate to dTMP and dihydrofolate. Because of its central role in the de novo synthesis of dTMP, this enzyme has been the target of a number of inhibitors with potential chemotherapeutic utility.[2] dTMP synthetase is a target for drugs such as 5-fluorouracil and 5-fluoro-2′-deoxyuridine (FdUrd); metabolism of these compounds

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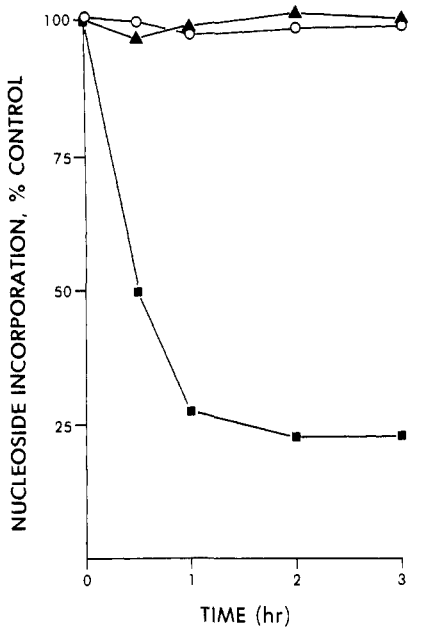

Figure 1. Effect of NO_2dUrd on incorporation of nucleosides into nucleic acids. Exponentially growing L1210 cells were harvested and resuspended in fresh media containing NO_2dUrd (0.6 μM). A parallel control culture was resuspended in media without NO_2dUrd. At the indicated times, an aliquot of the treated and control suspensions was withdrawn and placed in tubes containing either [6-^{3}H]dUrd (0.024 μM; ■), [5-^{3}H]Urd (0.062 μM; ○), or [*Me*-^{3}H]dThd (0.18 μM; ▲). Following this transfer, cells were incubated for 1 h at 37 °C. Incorporation of radioactivity into acid-soluble material was then determined as described under Experimental Section.

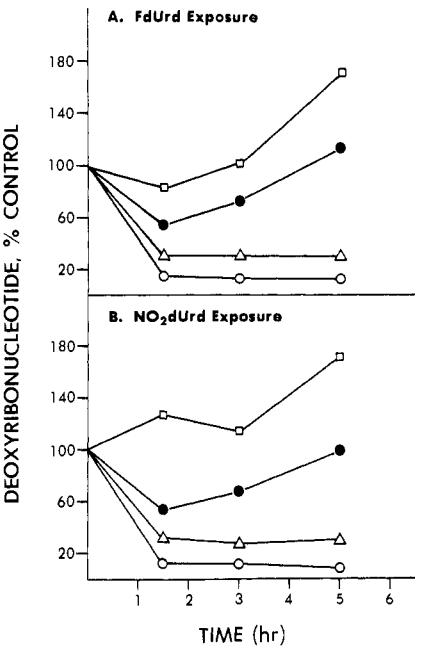

Figure 2. Deoxynucleoside content of the acid-soluble fraction of L1210 cells following exposure to FdUrd or NO_2dUrd. Exponentially growing L1210 cells were incubated with (A) FdUrd (10 nM) or (B) NO_2dUrd (0.6 μM). At the times indicated, aliquots of the culture were removed and analyzed for deoxyribonucleoside content (see Experimental Section): dCTP (●); dTTP (△); dATP (□); dGTP (○).

results in formation of the corresponding deoxyribonucleotide 5-fluoro-2′-deoxyuridylate (FdUMP), which forms a covalent complex with the enzyme with resultant cytotoxicity to proliferating cells. It has been demonstrated that 5-NO_2dUMP is a potent, mechanism-based inhibitor of dTMP synthetase;[3,4] after formation of a tight reversible complex, a covalent bond is formed between the 6-position of the heterocycle and a thiol group of the enzyme, which is believed to be a nucleophilic catalyst. Reports from this and other laboratories have demonstrated that the corresponding nucleoside, NO_2dUrd, is both a potent inhibitor of growth of cells in culture[5,6] and an active antiviral agent;[7] these effects have been attributed to inhibition of dTMP synthetase. In this paper we describe experiments that confirm the mechanism of action of NO_2dUrd; it is converted by dThd kinase to NO_2dUMP, which forms a tight complex with dTMP synthetase. In addition, results of in vivo testing of NO_2dUrd as an antineoplastic agent are reported.

Results and Discussion

NO_2dUrd is a very potent growth inhibitor of L1210 and S49 cells in culture, with EC_{50} values of 33 and 30 nM, respectively. The corresponding pyrimidine base, 5-nitrouracil (NO_2Ura), was not inhibitory at concentrations up to 0.3 mM, and S49/TK$^-$ cells, which are lacking dThd kinase, were not inhibited by 3 μM NO_2dUrd.[5] Thus, the inhibition observed with NO_2dUrd does not result from catabolism to NO_2Ura but rather requires conversion to NO_2dUMP by dThd kinase.

As previously observed,[5,6] studies of reversal of NO_2dUrd cytotoxicity by dThd and dUrd, as well as those examining dThd and dUrd uptake in the presence of NO_2dUrd into nucleic acids, show rather specific patterns. Growth inhibition by NO_2dUrd is greatly reduced in the presence of 10 μM dThd (EC_{50} > 3 μM) but only slightly affected by 10 μM dUrd (EC_{50} = 0.12 and 0.06 μM for L1210 and S49 cells, respectively). Further, treatment of L1210 cells with NO_2dUrd reduces the rate of dUrd incorporation into DNA but has no effect on dThd incorporation (Figure 1). Likewise, the drug has no effect on the incorporation of Urd into RNA (Figure 1). Taken together, these results strongly implicate dTMP synthetase as the target for NO_2dUrd action, and the remainder of what is described here demonstrates this point.

As a result of dTTP depletion and consequent allosteric effects on ribonucleotide reductase,[8,9] intracellular blockade of dTMP synthetase results in characteristic changes in the deoxyribonucleoside triphosphate (dXTP) pool sizes. This pattern is illustrated in Figure 2A, which shows the changes in dXTP's in L1210 cells following exposure to FdUrd at 20 times its EC_{50} (0.5 mM),[5] which, under the conditions used, has been demonstrated to afford specific inhibition of dTMP synthetase.[10] As shown, there is a rapid depletion of dTTP and dGTP levels, an initial fall of dCTP followed by recovery, and an expansion of the dATP pool; as expected, no changes were observed in the ribonucleoside triphosphate pools. Figure 2B shows that at 20 times its EC_{50} value, NO_2dUrd has an almost iden-

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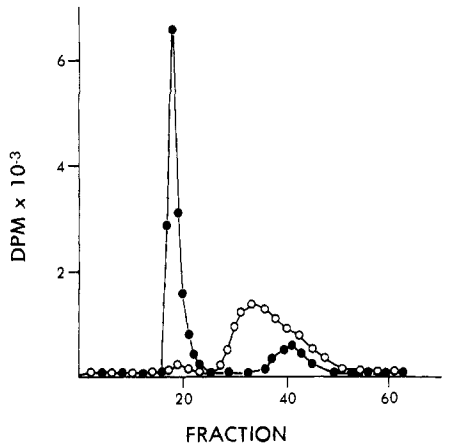

Figure 3. Chromatography of cell cytosol from L1210 cells exposed to [^{3}H]NO_2dUrd. Exponentially growing L1210 cells were incubated with [6-^{3}H]NO_2dUrd for 2 h at 37 °C, and cell cytosol was prepared as described under Experimental Section. Aliquots of cytosol were subjected to gel filtration on Sephadex G-25 immediately (●) or after heating at 65 °C for 20 min (○). The fractions were analyzed for radioactivity.

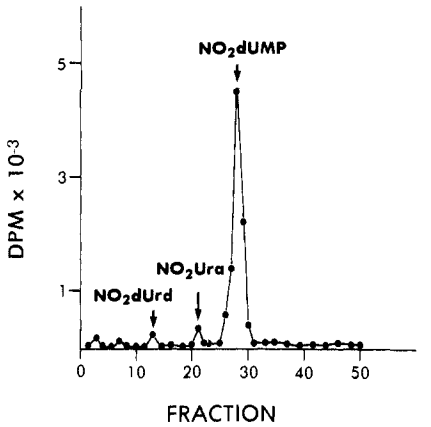

Figure 4. Identification of metabolites of [6-^{3}H]NO_2dUrd that are bound to macromolecules. The low-molecular-weight fraction obtained from Sephadex G-25 chromatography of cytosol heated at 65 °C for 20 min (see Figure 3) was analyzed by HPLC (see Experimental Section) in the presence of authentic standards.

tical effect on the dXTP pools in L1210 cells as does FdUrd; as with FdUrd, no changes were observed in the ribonucleoside triphosphates. These results provide additional strong evidence, albeit circumstantial, that NO_2dUrd is indeed causing inhibition of dTMP synthetase.

It has been possible to treat cells with FdUrd and subsequently isolate the covalent FdUMP–methylenetetrahydrofolate–dTMP synthetase complex formed within the cells.[10] The complex is formed in amounts equivalent to the enzyme content of cells, and upon heat-induced dissociation of this comples one can recover FdUMP. It has been shown that NO_2dUMP also forms a covalent complex with dTMP synthetase, which can be isolated by gel filtration and dissociated by denaturation.[3] Figure 3 illustrates the radioactivity profile obtained after gel filtration of cell cytosol from L1210 cells that had been incubated for 2 h at 37 °C with [6-^{3}H]NO_2dUrd. Two peaks of radioactive material were present: one corresponded to macromolecule-bound radioactivity and the other to radioactivity of low molecular weight. HPLC chromatography demonstrated that the latter was composed of unmetabolized NO_2dUrd, NO_2Ura, and a small amount of NO_2dUMP. The radioactivity in the macromolecular fraction represented 0.078 pmol/10^6 cells, which is in excellent agreement with the amount of dTMP synthetase (0.080 pmol/10^6 cells) in L1210 cells as determined by titration with [^{3}H]FdUMP.[10] Further, all the radioactive material bound to macromolecules was dissociated by heating (Figure 3); the released radioactive ligand was identified as NO_2dUMP by HPLC (Figure 4). These results provide conclusive evidence that incubation of L1210 cells with NO_2dUrd results in its enzymatic conversion to NO_2dUMP, with subsequent inhibition of dTMP synthetase by this deoxynucleotide. From these data, together with other studies described here, it may be concluded that this inhibition is responsible for the growth inhibitory effect of NO_2dUrd.

The properties of NO_2dUrd toward tissue culture cells suggest that it might have potential as an antineoplastic agent. Like FdUrd, NO_2dUrd is a potent and specific inhibitor of dTMP synthetase. Unlike FdUrd, the presence of cofactor is not required for enzyme inhibition,[3] and the corresponding pyrimidine base NO_2Ura—a catabolite of NO_2dUrd—is not cytotoxic toward cells, as is fluorouracil. The in vivo antineoplastic activity of NO_2dUrd was tested with mice bearing L1210 leukemia. Treatments were begun 24 h after implantation of 10^6 leukemia cells, and daily doses up to 400 mg/kg were administered for 6 consecutive days. At 400 (mg/kg)/day, only a 33% increase in the survival time of tumor-bearing mice was observed; even at this large dose of drug, toxic side effects manifested by weight loss or death did not occur. The relatively low in vivo activity of high doses of NO_2dUrd is attributed to its degradation by dThd phosphorylase, which is present in many tissues[11] and rapidly degrades NO_2dUrd to the nontoxic NO_2Ura.[12] It is impractical to consider larger bolus doses of NO_2dUrd, but this compound might prove more effective if administered as a continuous infusion or in combination with an inhibitor of dThd phosphorylase, should effective compounds become available.

Experimental Section

Chemicals. NO_2dUrd was prepared and purified by a reported procedure.[13] [6-^{3}H]NO_2dUrd (20 Ci/mmol) was prepared by nitration of [6-^{3}H]Ura (Moravek Biochemicals), followed by treatment with dUrd and nucleoside deoxyribosyltransferase as previously described.[3] FdUrd was obtained from the Division of Cancer Treatment, NCI. [6-^{3}H]dUrd (16 Ci/mmol), [*Me*-^{3}H]dThd (55 Ci/mmol), and [5-^{3}H]Urd (21 Ci/mmol) were obtained from Moravek Biochemicals. All other chemicals were of analytical grade.

Cell Culture. S49 and S-49/TK mouse lymphoma cells and L1210 mouse leukemia cells were maintained at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% heat-treated horse serum. The S-49/TK$^-$ strain (BU-S-M-bromodeoxyuridine-40-3) was selected for resistance to bromodeoxyuridine and was shown to be deficient in thymidine kinase activity.[14]

Growth Inhibition. Cells (6–8 × 10^4) were suspended in 1 mL of their original medium containing specified inhibitors and incubated at 37 °C for 48 to 72 h. Cell number was determined on a Coulter counter and maximally increased 10- to 20-fold over that inoculated. EC_{50} values refer to the concentration of inhibitor necessary to inhibit cell growth by 50% compared to controls grown under identical conditions, except that the inhibitor was omitted.

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Incorporation of Nucleosides into RNA and DNA. Three milliliters of an exponentially growing cell suspension (4–5 × 10^5 cells/mL) was treated with the appropriate radioactive nucleoside for 1 h at 37 °C. Cells were harvested by centrifugation (1000*g*, 5 min) at 4 °C and washed with 5 mL of ice-cold phosphate-buffered saline. Cells were mixed with 10 volumes of ice-cold 5% TCA, the precipitate was collected by suction onto 2.4-cm Whatman GF/C glass-fiber filters, and the filters were washed twice with 2.5 mL of ice-cold 5% TCA. The filters were then placed into glass scintillation vials to which was added 0.5 mL of Protosol (New England Nuclear, Boston, MA). After incubation of the tightly covered vials at 68 °C for 30 min, 0.05 mL of glacial acetic acid was added, followed by 10 mL of scintillation fluid, and the vials were counted.

Metabolism of [6-^{3}H]NO_2dUrd. Exponentially growing L1210 cells were harvested and resuspended at a density of 2 × 10^6 cells/mL in 3 mL of fresh media containing [6-^{3}H]NO_2dUrd (20 Ci/mmol; final concentration 165 nM). The cells were maintained at 37 °C in a CO_2 incubator for 2 h, at which time the cells were pelleted at 4 °C (1000*g*, 5 min) and washed twice with ice-cold phosphate-buffered saline. The cell pellet was suspended in 0.3 mL of this buffer, and a cell homogenate was prepared by sonication of the cell suspension (Brownwill Biosonik, PI = 40, 20 s). Cell debris was removed by centrifugation (4 °C, 1000*g* for 5 min). An aliquot of cytosol (0.2 mL) was applied to a Sephadex G-25 column (0.7 × 13 cm) equilibrated at 4 °C with 20 mM sodium phosphate (pH 7.3) and 10 mM β-mercaptoethanol. Column fractions (0.4 mL) were collected, and aliquots were analyzed for radioactivity. A clean separation was obtained between radioactivity bound to macromolecules eluting in the void volume and low-molecular-weight metabolites (see text). The pooled high-molecular-weight fraction was heated at 65 °C for 20 min and analyzed by repassage through Sephadex G-25. Fractions were collected and analyzed for radioactivity by liquid scintillation counting. The low-molecular-weight fraction from each Sephadex G-25 chromatography was pooled and taken to dryness by lyophilization; the residue was dissolved in water and analyzed by HPLC with a Lichrosorb RP-18 column (4.6 × 250 mm) with 5 mM $(n\text{-Bu})_4N^+HSO_4^-$, 5 mM potassium phosphate, pH 7.1, 20% MeOH. The retention volumes of authentic standards in this system are: NO_2dUrd, 7 mL; NO_2Ura, 12 mL; NO_2dUMP, 19 mL.

Nucleoside Triphosphate Pools. Exponentially growing L1210 cells were treated with specified amounts of NO_2dUrd or FdUrd. At intervals of 1.5, 3, and 5 h, acid-soluble fractions were prepared and analyzed for ribo- and deoxyribonucleoside triphosphates as previously described.[15]

Antineoplastic Activity in Vivo. Female CDH_1 mice were inoculated intraperitoneally with 0.1 mL of a suspension of L1210 cells (10^6 cells/mL). The cells were taken from a 7-day ascites tumor-bearing animal and were diluted with normal saline. After all of the animals had been inoculated, they were divided into groups of five, such that each group had approximately the same mean weight. Drugs were suspended or dissolved in saline containing 5% ethanol and 1 drop of 20% aqueous Tween-80 per 12 mL at the concentration used for the highest doses. Lower doses were obtained by serial dilution with saline. Drugs (in 0.5 mL) were injected ip on days 1 to 6 after tumor implantation. Control animals received 0.5 mL of saline. Each group of animals was weighed on the day following the last injection (day 7), and the difference in weight from the day of implantation (day 0) was noted and used as a measure of toxicity.

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Synthesis and Biological Evaluation of 6-Ethynyluracil, a Thiol-Specific Alkylating Pyrimidine

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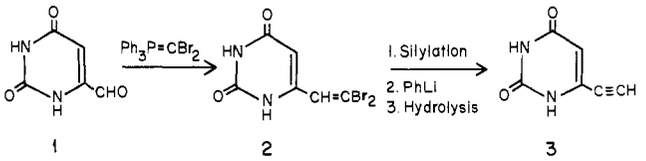
6-Ethynyluracil (**3**) was prepared by two different synthetic procedures. In one approach, 6-formyluracil was reacted with (dibromomethylene)triphenylphosphorane to give 6-(2,2-dibromovinyl)uracil (**2**), which was silylated and treated with phenyllithium to yield **3**. Alternatively, silylated 6-iodouracil was reacted with trimethylsilylacetylene in dry triethylamine in the presence of a palladium/copper catalyst to give 6-[(trimethylsilyl)ethynyl]uracil (**5**). Compound **5** was converted to **3** in refluxing methanol. At neutral pH, **3** reacted with thiols, such as glutathione, 2-mercaptoethanol, and L-cysteine, but did not react with glycine or L-lysine. This reaction was accompanied by a shift in the UV maximum of **3** from 286 nm to 321–325 nm. The reaction of **3** with 2-mercaptoethanol gave *cis*-6-[2-[(2-hydroxyethyl)-thio]vinyl]uracil as the predominant product. Compounds **2** and **3** inhibited the growth of leukemia L1210, B-16 melanoma, and lewis lung carcinoma cells at concentrations ranging from 1 × 10^{-6} to 2 × 10^{-5} M. As determined with L1210 cells, the inhibition of growth caused by **2** and **3** was not prevented by the natural pyrimidines, indicating that the agents do not act as antimetabolites.

Previous work in this laboratory on the development of new pyrimidine analogues and their nucleoside derivatives led to the synthesis of 5-ethynyluracil nucleosides,[1] which showed in vitro activity against leukemia L1210 cells at concentrations as low as 2 × 10^{-8} M. In a related effort, we have introduced the ethynyl group at the 6-position of uracil with the aim of generating an analogue of orotic acid. The synthesis and biological evaluation of 5-ethynyluracil nucleosides have also been carried out in various other laboratories,[2,3] and the preparation of 5-ethynylorotic acid

Scheme I



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