Irreversible Enzyme Inhibitors. 202.[1,2] Candidate Active-Site-Directed Irreversible Inhibitors of 5-Fluoro-2′-deoxyuridine Phosphorylase from Walker 256 Rat Tumor Derived from 1-Benzyl-5-(3-ethoxybenzyl)uracil[3]

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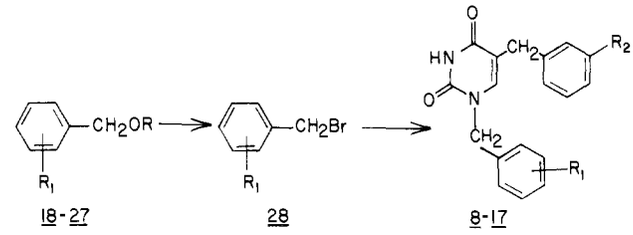
Six candidate irreversible inhibitors of uridine–deoxyuridine phosphorylase (EC 2.4.2.3) from Walker 256 rat tumor were synthesized. These compounds connect a terminal sulfonyl fluoride group to the 1-benzyl moiety of 1-benzyl-5-(3-ethoxybenzyl)uracil (**9**). Although none of the compounds were irreversible inhibitors, the four 3-[(fluorosulfonyl)benzamido] analogues (**14–17**) of **9** were good reversible inhibitors of the enzyme which were complexed from 125- to 360-fold better than the substrate, 5-fluoro-2′-deoxyuridine.

There are two enzymes in mammalian tissues that start the detoxification of 5-fluoro-2′-deoxyuridine (FUDR) by cleavage to 5-fluorouracil (FU) and 2-deoxy-α-D-ribofuranosyl phosphate; these are uridine–deoxyuridine phosphorylase (EC 2.4.2.3)[5–7] and thymidine phosphorylase (EC 2.4.2.4).[5–8] Walker 256 rat tumor contains an FUDR cleaving enzyme that apparently is only uridine–deoxyuridine phosphorylase.[9] The latter can be inhibited by 6-(aralkylamino)uracils, which can complex as much as fourfold better than the substrate FUDR.[10] A much stronger interaction was observed with 1-aralkyl derivatives of uracil; the latter could complex as much as 180-fold better than FUDR.[11] In an extensive study of 5-substituted uracils, 5-(3-$CH_3CH_2OC_6H_4CH_2$)- and 5-(3-$C_6H_5CH_2OC_6H_4CH_2$)-substituted compounds were found to bind 300- and 800-fold better than the substrate.[12] The bulk tolerance for a 1-benzyl group on 5-benzyluracil[12] suggested that active-site-directed irreversible inhibitors[13] of this FUDR phosphorylase could be constructed by bridging a covalent bond forming group from the 1-benzyl moiety. The synthesis and enzyme evaluation of such candidate active-site-directed irreversible inhibitors of the uridine-deoxyuridine phosphorylase from Walker 256 tumor are the subject of this paper.

Chemistry. The 1-substituted uracils **8–17** were prepared in two steps as outlined in Scheme I. The starting methyl benzyl ethers (**18**, **20**, **21**, and **24–27**) (Table III) and benzyl alcohols (**19**, **22**, and **23**) were prepared by acylation of 3- or 4-aminobenzyl methyl ether[14,15] or 3-aminobenzyl alcohol[16] with the appropriate benzoyl chloride (for **18–23**), 4-nitrophenyl *N*-[4-(fluorosulfonyl)-phenyl]carbamate[17] (for **24** and **26**), or *m*-(fluorosulfonyl)phenyl isocyanate (for **25** and **27**). The intermediate benzyl bromides were prepared from **18–27** by treatment with anhydrous 30–32% HBr–AcOH to give solids, which were dried and used without further purification. The 4-benzamides **24** and **25** failed to give stable benzyl bromides with anhydrous 30–32% HBr–AcOH at reflux (5 min), ambient temperature (12 h), or with anhydrous HBr in THF (1 h). A polar product was obtained which stayed at the origin on TLC in solvent B.

The trimethylsilyl method[18–21] for selective alkylation of uracils at the 1 position was successfully adapted to the synthesis of the compounds in Table II. The bis(trimethylsilyl) ethers of **3** and **4** were formed in refluxing hexamethyldisilazane and reacted without isolation with the appropriate benzyl bromide **28** in acetonitrile. After an extended reflux and a hydrolytic workup, good yields of **8–17** were obtained (Table II). Although these benzyl bromides reacted at a satisfactory rate, benzyl chloride was unreactive.

Scheme I



Enzyme Results

The discovery that 5-benzyluracils, such as **1–4** (Table

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(1) This work was generously supported by Grant CA-08695 from the National Cancer Institute, U.S. Public Health Service.
(2) The paper by M. H. Doll and B. R. Baker, *J. Med. Chem.*, **19**, 1079 (1976), is considered to be paper 201 in this series.
(3) Abstracted from the Ph.D. Dissertation submitted by J. L. Kelley to the Department of Chemistry of the University of California at Santa Barbara, March 1970.
(4) Deceased October 19, 1971.
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(8) For a discussion of the chemotherapeutic use for inhibitors of these detoxification enzymes for FUDR, see B. R. Baker, *J. Med. Chem.*, **10**, 297 (1967).
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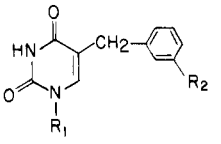
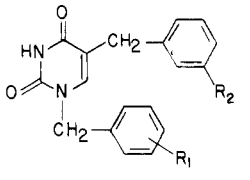
Table I. Reversible and Irreversible Inhibition [a] of Walker 256 FUDR Phosphorylase by Compounds 1-17



no.	R_1	R_2	inhibition: reversible: I_{50}, [b] μM	inhibition: irreversible: [c] V_0/V_1
1	H	H	5.3 [d]	
2	H	OCH_3	1.9 [d]	
3	H	OCH_2CH_3	1.4 [d]	
4	H	$OCH_2C_6H_5$	0.50 [d]	
5	CH_3	H	6.2 [d]	
6	$CH_2C_6H_5$	H	2.6 [d]	
7	$(CH_2)_3OC_6H_5$	H	5.7 [d]	
8	$CH_2C_6H_5$	$OCH_2C_6H_5$	2.5	
9	$CH_2C_6H_5$	OCH_2CH_3	1.2	
10	$CH_2C_6H_4$-4-$NHCOC_6H_5$	OCH_2CH_3	3.8	
11	$CH_2C_6H_4$-3-$NHCOC_6H_5$	OCH_2CH_3	1.2	1.2
12	$CH_2C_6H_4$-4-$NHCOC_6H_4SO_2F$-4	OCH_2CH_3	15	1.0
13	$CH_2C_6H_4$-4-$NHCOC_6H_4SO_2F$-3	OCH_2CH_3	33	1.0
14	$CH_2C_6H_4$-3-$NHCOC_6H_4SO_2F$-4	OCH_2CH_3	2.5	1.0
15	$CH_2C_6H_4$-3-$NHCOC_6H_4SO_2F$-3	OCH_2CH_3	3.2	1.1
16	$CH_2C_6H_4$-3-$NHCONHC_6H_4SO_2F$-4	OCH_2CH_3	1.9	1.1
17	$CH_2C_6H_4$-3-$NHCONHC_6H_4SO_2F$-3	OCH_2CH_3	1.1	1.2

[a] The technical assistance of Maureen Baker, Julie Leseman, and Janet Wood is acknowledged. [b] I_{50} = concentration for 50% inhibition when assayed with 400 μM FUDR in pH 5.9 arsenate-succinate buffer containing 10% Me_2SO as previously described.[8,10] [c] The enzyme was preincubated at ambient temperature with 20 μM inhibitor for 20 min in pH 5.9 arsenate-succinate buffer containing 10% Me_2SO. Then the assay mixture was extracted with 1-octanol to remove inhibitor, and the remaining enzyme was assayed as described under Experimental Section. V_0 = velocity without inhibitor; V_1 = velocity with inhibitor; a V_0/V_1 ratio of 2 would be indicative of 50% inhibition of the enzyme under the preincubation conditions. [d] Data from ref 12.

Table II. Physical Properties of Compounds 8-17



no.	R_1	R_2	reaction time, [a] h	yield, %	mp, °C	formula [b]
8	H	$OCH_2C_6H_5$	40	41	116-119 [c]	$C_{25}H_{22}N_2O_3$
9	H	OCH_2CH_3	80	57 [d]	136-138	$C_{20}H_{20}N_2O_3$
10	4-$NHCOC_6H_5$	OCH_2CH_3	19	20	255-258 [e]	$C_{27}H_{25}N_3O_4$
11	3-$NHCOC_6H_5$	OCH_2CH_3	41	51 [e]	212-213	$C_{27}H_{25}N_3O_4$
12	4-$NHCOC_6H_4SO_2F$-4	OCH_2CH_3	24	71	190-194 [d]	$C_{27}H_{26}FN_3O_6S$
13	4-$NHCOC_6H_4SO_2F$-3	OCH_2CH_3	40	61	220-222 [d]	$C_{27}H_{26}FN_3O_6S$
14	3-$NHCOC_6H_4SO_2F$-4	OCH_2CH_3	43	21	198-220 [f]	$C_{27}H_{26}FN_3O_6S$
15	3-$NHCOC_6H_4SO_2F$-3	OCH_2CH_3	28	37 [d]	102-108	$C_{27}H_{26}FN_3O_6S$
16	3-$NHCONHC_6H_4SO_2F$-4	OCH_2CH_3	40	60 [d]	140-143	$C_{27}H_{27}FN_4O_6S$
17	3-$NHCONHC_6H_4SO_2F$-3	OCH_2CH_3	28	66	118-130 [g]	$C_{27}H_{27}FN_4O_6S$

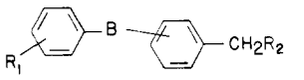
[a] All compounds were prepared by method E. The intermediate benzyl bromides were prepared by method D. [b] All compounds were analyzed for C, H, and N. [c] Recrystallized from 2-PrOH. [d] Recrystallized from EtOH. [e] Recrystallized from EtOH-MeOEtOH. [f] Recrystallized from MeOEtOH. [g] Recrystallized from EtOH-H_2O.

I), were potent reversible inhibitors of the uridine–deoxyuridine phosphorylase from Walker 256 rat tumor[12] opened an avenue for irreversible studies on this enzyme. That the 1-H of 1 was not complexed to the enzyme was shown by the fact that the 1-CH_3 derivative **5** was equally active.[12] Furthermore, when large groups, such as 1-$CH_2C_6H_5$ (**6**) or 1-$(CH_2)_3OC_6H_5$ (**7**) were substituted on **1**, no loss in binding occurred, indicating that there was bulk tolerance for large groups on the 1 position of 5-benzyluracil (**1**). When **4**, which was tenfold more inhibitory than **1**, was substituted on the 1 position with a benzyl group, the product (**8**) was fivefold less active. This loss may be due to intramolecular steric interference between the two aryl groups, thus preventing the most favorable conformation for binding to the enzyme. Since there was only limited tolerance for the 1-benzyl group of **8**, there probably would be even less tolerance for a 1-benzamidobenzyl moiety on **4**.

When the next best inhibitor, **3**, was substituted with a 1-benzyl group to give **9**, no loss of inhibition occurred. There was also bulk tolerance on the 1-benzyl moiety of **9** for a 3-$NHCOC_6H_5$ group (**11**), and only a threefold loss in binding occurred with the 4-$NHCOC_6H_5$ group (**10**).

The bulk tolerance for the 1-(benzamidobenzyl) moiety on **3** suggested that active-site-directed irreversible inhibitors of this FUDR phosphorylase from Walker 256 could be constructed by having the benzamido moiety bear a terminal sulfonyl fluoride.[22] The latter group has been

Table III. Physical Constants of Compounds 18-27



no.	R_1	B	R_2	method	yield, %	mp, °C	formula [a]
18	H	CONH-4	OCH_3	A [b]	67	98-100 [c,d]	
19	H	CONH-3	OH	B [e]	76	112-113 [f,g]	
20	4-SO_2F	CONH-4	OCH_3	A [b]	89 [h]	188-189	$C_{15}H_{14}FNO_4S$
21	3-SO_2F	CONH-4	OCH_3	A [b]	63	122-123 [h]	$C_{15}H_{14}FNO_4S$
22	4-SO_2F	CONH-3	OH	A [e]	84 [c]	168-171	$C_{14}H_{12}FNO_4S$
23	3-SO_2F	CONH-3	OH	A [e]	42 [i]	151-152	$C_{14}H_{12}FNO_4S$
24	4-SO_2F	NHCONH-4	OCH_3	C [b,j,k]	81	184-185 [h]	$C_{15}H_{15}FN_2O_4S$
25	3-SO_2F	NHCONH-4	OCH_3	A [b,k,l]	89	142-143 [h]	$C_{15}H_{15}FN_2O_4S$
26	4-SO_2F	NHCONH-3	OCH_3	C [j,k,m]	83 [h]	179-181	$C_{15}H_{15}FN_2O_4S$
27	3-SO_2F	NHCONH-3	OCH_3	A [k-m]	83 [h]	133-134	$C_{15}H_{15}FN_2O_4S$

[a] All new compounds were analyzed for C, H, and N. [b] For the starting amine, see ref 14. [c] Recrystallized from EtOH-H_2O. [d] Mp 111-113 °C reported for this compound by a different method by G. Romeo, *Gazz. Chim. Ital.*, 35I, 111 (1905). [e] For the starting amine, see ref 16. [f] Recrystallized from C_6H_6. [g] Mp 115 °C reported for this compound by a different method by K. Auwers and K. Sonnenstuhl, *Chem. Ber.*, 37, 3937 (1904). [h] Recrystallized from EtOAc-petroleum ether (bp 60-110 °C). [i] Recrystallized from PrOH-H_2O. [j] For the starting carbamate, see ref 17. [k] Et_3N was omitted. [l] The commercially available isocyanate was used. [m] For the starting amine, see ref 15.

found to be particularly effective for formation of a covalent bond with an enzyme outside its active site, presumably with an appropriately located serine.[22,23] Since the position of such an hydroxyl on the phosphorylase is unknown, a random search was made by positioning the sulfonyl fluoride at the meta and para positions of **10** and **11** to give **12-15**. The ureido bridge was utilized in **16** and **17**, since the additional NH positions the sulfonyl fluoride differently from the carboxamido of **14** and **15**.

Although the meta-substituted derivatives (**14-17**) were nearly as effective as **9** when measured as reversible inhibitors (Table I), the two para compounds (**12** and **13**) sustained 12- and 27-fold losses in their affinity for the enzyme. This was somewhat unexpected, since from **10** there appeared to be sufficient bulk tolerance for large groups on the para position of **9**. The higher I_{50} values in the case of **12** and **13** may be indicative of the close proximity to the enzyme surface of the benzamido moiety of **10**, resulting in limited bulk tolerance for the sulfonyl fluoride group.

When **12-17** were measured for their ability to irreversibly inhibit the Walker 256 FUDR phosphorylase, none were inhibitory when preincubated with the enzyme for 20 min (Table I). Since the noncovalently bound inhibitor was removed from the assay mixture, a V_0/V_i ratio of 2 would be indicative of 50% inhibition. Although **12-17** were inactive as irreversible inhibitors, the loss in reversible binding sustained by the para-substituted fluorosulfonyl benzamides **12** and **13** suggests that this region of the inhibitors is in contact with the enzyme. This is an essential, although not sufficient, criterion for covalent bond formation between the sulfonyl fluoride and a nucleophilic group on the enzyme surface. Further searches for irreversible inhibitors could be carried out by varying the bridging length and the nature of the bridge.

Several rat tumors, including Walker 256, have only uridine-deoxyuridine phosphorylase and no thymidine phosphorylase.[9] Since rat liver contains both enzymes, increased efficacy of in vivo FUDR treatment of these rat tumors should be seen in the presence of reversible uridine-deoxyuridine phosphorylase inhibitors, such as **1-11**. FUDR will then not be detoxified by cleavage to FU in the tumor, whereas rat liver will continue to cleave FUDR with thymidine phosphorylase. Even if such an experiment were successful, it is unlikely that it could be applied to human tumors. Of a number of human tumors that have been examined,[9,24,25] all contained thymidine phosphorylase and possibly unknown amounts of uridine-deoxyuridine phosphorylase. Any tumor containing thymidine phosphorylase could still cleave FUDR, since this enzyme is not inhibited by the potent inhibitors in Table I;[26] however, any human tumor containing only uridine-deoxyuridine phosphorylase should respond like Walker 256.[27]

Recent interest in the pyrimidine nucleoside cleaving enzymes in mammalian cells[25,27,28] has prompted the publication of these studies, which were delayed due to the untimely death of Dr. B. R. Baker.[4]

Experimental Section

Each compound had IR and UV spectra compatible with their assigned structures and each moved as a single spot on TLC on Brinkman silica gel GF with the indicated solvent system: (A) C_6H_6-EtOH, 3:1; (B) EtOAc-petroleum ether (bp 60-110 °C), 1:2; (C) $CHCl_3$-EtOH, 1:1; (D) C_6H_6-dioxane-AcOH, 90:25:4; (E) EtOAc-petroleum ether (bp 60-110 °C), 1:1; (F) AcOH-C_6H_6, 1:5; (G) EtOAc-petroleum ether (bp 60-110 °C), 1:18. Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. The analytical samples gave combustion values for C, H, and N within 0.4% of theory. The microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

3-[4-(Fluorosulfonyl)benzamido]benzyl Alcohol (22). Method A. To a stirred solution of 1.23 g (10 mmol) of 3-aminobenzyl alcohol[16] and 1.11 g (11 mmol) of Et_3N in 10 mL of dioxane was added a solution of 2.23 g (10 mmol) of 4-(fluorosulfonyl)benzoyl chloride in 10 mL of dioxane. After 1 h, the resultant mixture was diluted with 300 mL of H_2O and acidified to pH 1 with 1 N HCl. The product was collected, washed with H_2O, and recrystallized from EtOH-H_2O: yield 2.61 g (84%) of white granules; mp 168-171 °C; IR (Nujol) 3300 (broad, NH, OH); 1645, 1560, 1300 (amide); 1410, 1210 (SO_2F); 1030 (CO) cm^{-1}. See

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(28) D. V. Santi, *J. Med. Chem.*, **23**, 103 (1980).

Table III for additional data and other compounds prepared by this method.

In **Method B**, the product oiled out of solution and was extracted with two 25-mL portions of $CHCl_3$. The combined extracts were washed with 25 mL of brine, dried ($MgSO_4$), and spin evaporated in vacuo to give a clear syrup, which solidified when left overnight. In **Method C**, DMF was used as the solvent; the crude product was dissolved in a minimum of Me_2CO, diluted with 300 mL of ice–H_2O, and basified to pH 7–8 with 5% $NaHCO_3$. The product was collected and washed with H_2O, and the dissolution–precipitation was repeated twice more when the yellow color of 4-nitrophenol was no longer present. See Table III for compounds prepared by these methods.

3-[4-(Fluorosulfonyl)benzamido]benzyl Bromide. Method D. A mixture of 0.449 g (1.4 mmol) of **22** and 5 mL of 30% anhydrous HBr–AcOH was heated at 100 °C for 10 min. The solution was cooled on an ice bath and then diluted with ice–H_2O. The product was collected, washed with H_2O, dried over $CaSO_4$, and finally over P_2O_5 under low vacuum: yield 0.520 g (96%) of a white powder, which was homogeneous by TLC (B) and gave a positive test for active halide.[29] This material was used without further purification.

5-(3-Ethoxybenzyl)-1-[4-[3-(fluorosulfonyl)benzamido]benzyl]uracil (13). Method E. A mixture of 0.246 g (1.0 mmol) of **3**, 5 mL of hexamethyldisilazane, and 0.2 mL of chlorotrimethylsilane with protection from moisture was refluxed with stirring for 4 h, during which dissolution occurred. To the cooled solution was added a dispersion of 0.560 g (1.5 mmol) of 4-[3-(fluorosulfonyl)benzamido]benzyl bromide in 5 mL of acetonitrile. The mixture was refluxed with stirring for 40 h, cooled, and spin evaporated in vacuo, and the residue was dissolved in 5 mL of hot EtOH. This solution was cooled and spin evaporated in vacuo, and the residue was triturated with 5 mL of ice–H_2O. The product was collected, washed with H_2O, and recrystallized from EtOH: yield 0.195 g (36%); mp 219–222 °C. Evaporation of the mother liquors afforded an additional 0.132 g (total 61%), mp 213–219 °C. Several addition recrystallizations gave the analytical sample as white granules: mp 220–222 °C; UV (EtOH) λ_{max} 280 nm; UV λ_{max} pH 13, 278 nm; IR (Nujol) 3280 (NH), 1685, 1660, 1600, 1530 (NHC=O, C=N, C=C), 1410, 1210 (SO_2F), 1255 (COC) cm^{-1}.

Irreversible Inhibition of FUDR Phosphorylase. The irreversible assay was carried out on twice the scale used for the reversible assay.[8] Five pairs of tubes were placed in a rack; the back tubes served as zero-time tubes. In each tube was placed 5.00 mL of the assay mix and Me_2SO or a Me_2SO-inhibitor solution, such that the final inhibitor concentration was 20 μM. After 20 min, 3.0 mL of 1-octanol was added to tube 1 of the back (zero time) tubes and mixed on a Vibro Jr. Mixer for 30 s. Then, tubes 2–5 of the back (zero time) tubes and tubes 1–5 of the front tubes were treated similarly. The tubes were centrifuged for 3 min, the 1-octanol layer was removed, and the extraction was repeated with 3.0 mL of fresh 1-octanol. After the second extraction had been centrifuged and removed, 500 μL of the aqueous layer from each tube was transferred to a new set of five paired tubes. (The outside of the pipet was wiped dry when delivering to the new tubes, and care was taken to avoid getting octanol in the pipet.) To each of the five new back (zero time) tubes was added 500 μL of 5% aqueous trichloroacetic acid, and the contents were mixed. To the new front tubes was added 50 μL of 4 mM FUDR at 30-s intervals, and the contents of each tube were mixed after each addition. Then, 50 μL of 4 mM FUDR was added to the new back (zero time) tubes, and the contents were mixed. After the incubation period, 500 μL of 5% aqueous trichloroacetic acid was added to each of the front tubes. All of the tubes were centrifuged for 5 min, and the solutions were then assayed as for the reversible assay.[8]

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(29) B. R. Baker, D. V. Santi, J. K. Coward, H. S. Shapiro, and J. H. Jordaan, *J. Heterocycl. Chem.*, 3, 425 (1966).

Inhibitors of Adenosine Deaminase. Studies in Combining High-Affinity Enzyme-Binding Structural Units. *erythro*-1,6-Dihydro-6-(hydroxymethyl)-9-(2-hydroxy-3-nonyl)purine[1] and *erythro*-9-(2-Hydroxy-3-nonyl)purine[2]

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erythro-1,6-Dihydro-6-(hydroxymethyl)-9-(2-hydroxy-3-nonyl)purine (4) was synthesized as a potential adenosine deaminase inhibitor, which combines in a single molecule two structural moieties, each of which possesses high affinity to a different region of the enzyme, the catalytic region and an auxiliary binding region which is specific for *erythro*-9-(2-hydroxy-3-nonyl)adenine (1). The potency of 4 ($K_i = 1.2 \times 10^{-5}$ M) is about one-seventeenth that of *erythro*-9-(2-hydroxy-3-nonyl)purine (2; $K_i = 6.8 \times 10^{-7}$ M), which contains only one high-affinity moiety. The mutually interfering rather than reinforcing effects of the two moieties may indicate the lack of simultaneous binding and thus provide insight into the relative geometry of the two binding regions of the enzyme.

Potent inhibitors of adenosine deaminases (ADA), which catalyze the hydrolytic N^6-deamination of adenosine, 2′-deoxyadenosine, and related nucleosides, are of interest as possible medicinal agents. Thus, in addition to its potentiating effect on nucleoside-type antitumor or antiviral agents, pentostatin[3] has generated interest for possible utility in immune modulation and lymphocyte control, as in human T-cell malignancies.[4,5]

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(1) [(*R**,*S**)-(±)]-β-Hexyl-1,6-dihydro-6-(hydroxymethyl)-α-methyl-9*H*-purine-9-ethanol.
(2) (*R**,*S**)-β-Hexyl-α-methyl-9*H*-purine-9-ethanol.
(3) Woo, P. W. K.; Dion, H. W.; Lang, S. M.; Dahl, L. F.; Durham, L. J. *J. Heterocycl. Chem.* **1974**, *11*, 641.

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