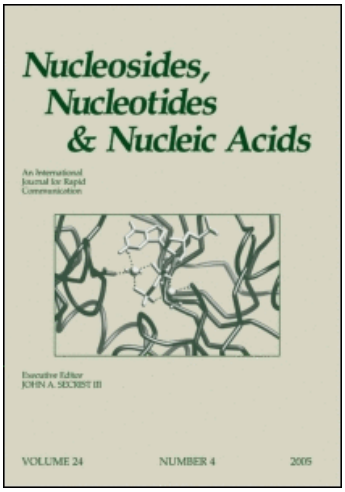

Nucleosides, Nucleotides and Nucleic Acids

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THE SYNTHESIS AND BIOLOGICAL ACTIVITY OF CERTAIN 4'-THIONUCLEOSIDES

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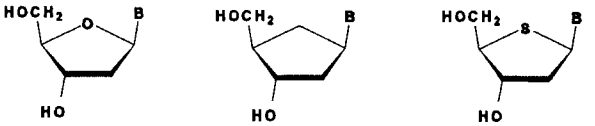
Abstract: Results are presented on the synthesis and biological activity of several types of 4'-thionucleosides as potential anticancer agents. Detailed studies on the mechanism of action of 4'-thiothymidine are also presented.

The search for nucleotide-based anticancer agents began over forty years ago, and has resulted in the development of a number of drugs used in the treatment of various types of cancers. These drugs include 5-fluorouracil, 6-mercaptopurine, 6-thioguanine, 1-β-D-arabinofuranosylcytosine, 9-(β-D-arabinofuranosyl)-2-fluoroadenine (given as the 5'-monophosphate), and 2-chloro-2'-deoxyadenosine. These drugs in general are most effective against leukemias, lymphomas, and other faster-growing cancers, and they are important weapons in the arsenal of antimetabolites used in cancer treatment.

Our approach for many years has been to focus on structural changes in nucleosides that will enhance selectivity while still allowing them to be metabolized to phosphate derivatives. Nucleosides with favorable alterations may have effects on the metabolic pathways leading to nucleic acids and/or on the proper utilization of nucleic acids after incorporation of an altered constituent. We have found that it is desirable to have as few changes as possible in a nucleoside structure in order to facilitate its interaction with the enzymes along the nucleic acid metabolic pathways, although the nature of the changes can be equally important.

One type of structural modification that we have pursued would confer on the nucleoside stability toward phosphorylase degradation, with the goals of preventing base cleavage as well as increasing the half-lives of the phosphorylated metabolites. The initial series of compounds of

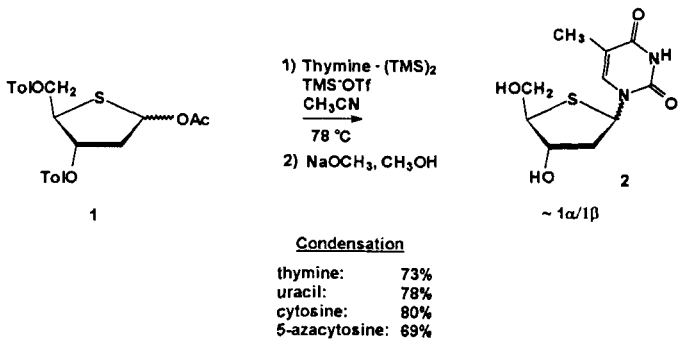
this type were carbocyclic nucleosides, where the furanose ring oxygen is replaced by a carbon. While many of these compounds have had interesting biological activity, only a very few compounds have shown selectivity in animal models for anticancer activity. An examination of the literature uncovered the fact that the substitution of a sulfur in place of the furanose ring oxygen would also confer stability to phosphorylase cleavage, at least in the specific example of 4'-thioinosine and purine nucleoside phosphorylase.[1] The relatively modest structural changes brought about by such a substitution also suggested that this direction might be attractive, and computer-generated molecular models (Sybyl) showed a close similarity between normal nucleic acids and those with the 4'-oxygen replaced with sulfur. We therefore set out to prepare a series of 4'-thionucleosides as potential anticancer agents utilizing our current knowledge of desirable bases and carbohydrates.



Specifically, we set out to synthesize drug targets that had 2-deoxy-4-thioribo- and arabinofuranose as their carbohydrate moieties, with certain purine and pyrimidine bases attached in the normal manner. Later, we have also included other carbohydrates, such as certain examples with the L configuration, based upon the more recent observations that certain L-nucleosides can be converted to the triphosphates and exert antiviral effects.[2-4] Some of the synthetic chemistry leading to certain of these target structures, and the biological activity of several target compounds will be covered, with a focus on the mechanism of action of 4'-thiothymidine, a compound we have studied in considerable detail.

Chemistry

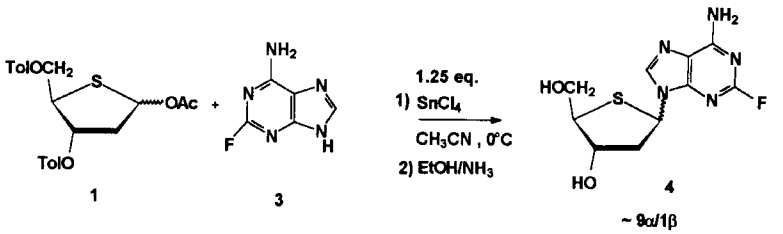
Some years ago pioneering work in the synthesis of 4'-thionucleosides was conducted in several laboratories.[5-10] Most of the effort was directed toward ribonucleosides, but one 2'-deoxynucleoside[9] and some arabinonucleosides[10] were also reported. We were able to prepare 1-*O*-acetyl-2-deoxy-3,5-di-*O*-*p*-toluoyl-4-thio-D-*erythro*-pentofuranose (**1**) by a modification of published route leading to the unblocked material.[11] The conversion of **1** to both pyrimidine[12] and purine 2'-deoxy-4'-thionucleosides has been accomplished. Examples in the pyrimidine series are shown in Scheme 1. Condensation conditions for pyrimidines utilized the silylated base in



Scheme 1

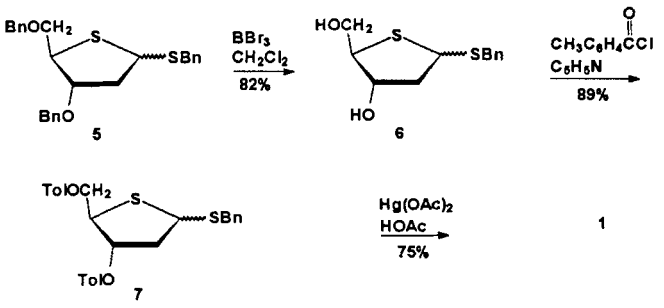
acetonitrile in the presence of trimethylsilyl trifluoromethanesulfonate at low temperature, and gave the yields noted in Scheme 1 as a *ca.* 1:1 α,β mixture. Deblocking with sodium methoxide in methanol produced the target nucleosides in yields around 80%. Similar sequences were used for uracil, cytosine and 5-azacytosine. For the latter compound, reduction with sodium borohydride prior to final deprotection was required in order to obtain the more stable 5,6-dihydro product. Separations of the α,β anomeric mixtures generally proved easier at the blocked nucleoside stage, though in the cytosine case we had to convert the blocked β-2'-deoxy-4'-thio uridine compound to the corresponding cytidine compound via the triazolyl intermediate.

For the purine nucleosides, a different set of condensation conditions were required to obtain good yields of nucleosides. The same carbohydrate precursor was treated with the appropriate purine base in acetonitrile at 0°C in the presence of stannic chloride to afford 70-80% yields of the blocked purine nucleosides, as shown in Scheme 2 for 2-fluoroadenine. Deblocking with ethanolic ammonia provided the free nucleoside. In all cases the ratios obtained were on the order of 9:1 in favor of the α isomer, using a variety of different catalysts and solvents. Examples of other bases that can be used are 6-chloropurine and 2,6-dichloropurine. In this latter case, conversion to the corresponding 2-chloroadenine, diaminopurine, and guanine nucleosides can be accomplished by conventional means. The problem of obtaining significant quantities of β anomer in the 2'-deoxy series has caused us to consider a number of alternatives. One of these, the production of 2'-deoxy compounds from the corresponding ribofuranosyl derivatives, is presented later.

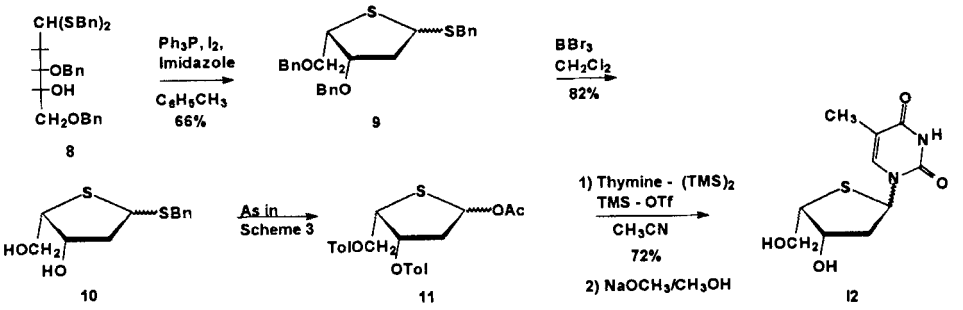


Scheme 2

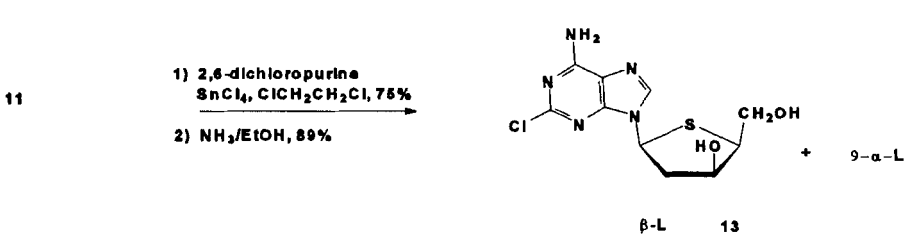
Other investigators have also recently pursued 4'-thionucleosides and the requisite 4-thiosugars, and several good routes to those sugars have been developed. The Walker laboratory developed a route beginning with a 2-deoxyribofuranose derivative, going through the dithioacetal, and involving a double inversion at C-4 to incorporate sulfur while retaining the original configuration.[13] More recently, the Uenishi laboratory has developed an interesting route to 2-deoxy-4-thio-D-*erythro*-pentofuranoses by going through epoxide and episulfide intermediates.[14] For our purposes, the Walker route appeared particularly suitable, and we have adapted it to generate a variety of 4'-thionucleoside precursors. The last few steps in the conversion of an intermediate in the Walker sequence to **1** (Schemes 1 and 2) is shown in Scheme 3. A comparison of the compound made by the earlier route and this one showed it to be the same material. During the course of this investigation, another route was published purporting to prepare a 2-deoxy-4-thioribofuranose precursor that was then converted into a thymine nucleoside.[15] This route claimed that a single reagent system effected both inversions at C-4 of a 2-deoxyribose intermediate to generate a blocked 2-deoxy-4-thioribofuranose precursor. Our investigation of the route showed that it did not, in fact, generate 4'-thiothymidine, but rather the C-4' epimer. This discovery opened up a simple route to a variety of α–L-*lyxo* nucleosides, exemplified by the thymine nucleoside shown in Scheme 4.[16] With this precursor, the α/β ratio is also about 1:1 in the pyrimidine series, and separation of the isomers is best carried out at the blocked nucleoside stage. The reagent system used, triphenylphosphine, iodine, and imidazole, has proven to be extremely effective in bringing about a single inversion, and we now use it routinely for this purpose. The same scheme can obviously be used for the synthesis of β-L-nucleosides, as well, and we have made a series of these compounds with purine bases, exemplified by the 2-chloroadenine nucleoside shown in Scheme 5.



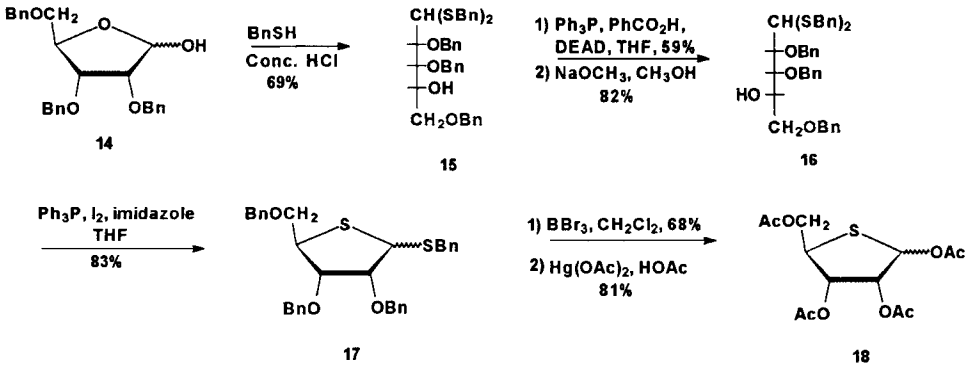
Scheme 3



Scheme 4



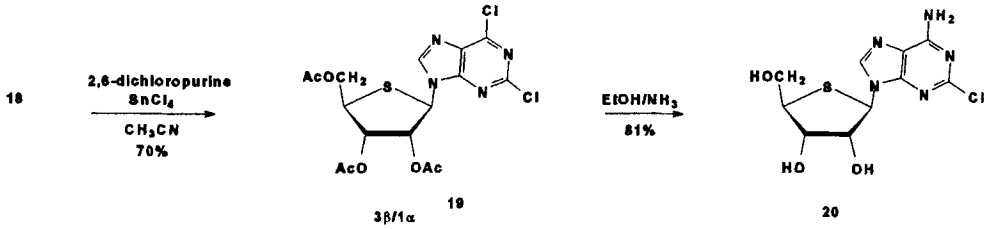
Scheme 5



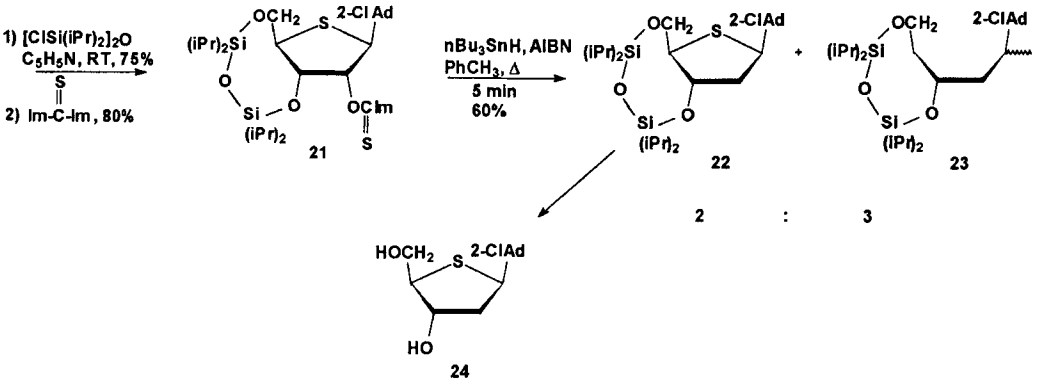
Scheme 6

Because of the low yields thus far encountered in the synthesis of purine 2'-deoxy-4'-thionucleosides, which included many of our desired targets, we elected to pursue their synthesis from the *ribo* nucleosides. The route that we used for generation of a *ribo* sugar is shown in Scheme 6.[17] This eight step route can be carried out on large scale to produce multigram quantities of tetra-*O*-acetyl-4-thio-D-ribofuranose (**18**) with good yields throughout the sequence. Utilization of **18** to prepare 2-chloro-4'-thioadenosine is shown in Scheme 7. As expected, the predominant product is the β anomer, although some α anomer, readily separated at the blocked nucleoside stage, is also formed. Conversion of **20** to the corresponding 2'-deoxynucleoside by deoxygenation has been carried out by a standard procedure as shown in Scheme 8.[17] The low yield in the deoxygenation step is attributable to the competition between loss of the 2'-group and removal of the 4'-sulfur, which produces a product consistent by mass spectral analysis with structure **23**. Removal of the silyl blocking group is readily accomplished with fluoride ion to produce **24**. While this route will provide the desired purine 2'-deoxy-4'-thionucleosides, it is still not an optimum approach to the β anomers. Further efforts to solve this problem are in progress.

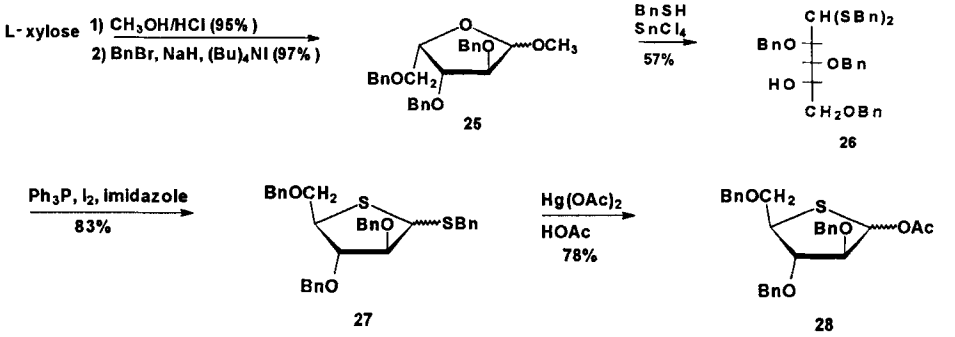
Another one of our target series, as noted earlier, is the 4'-thioarabinofuranosyl nucleosides containing selected purines and pyrimidines. We have prepared the precursor sugar **28** by two different methods. The first method employed an arabinose derivative, and involved a double inversion at C-4 for the conversion to the 4-thiosugar. While this route succeeded, one of the steps was quite low yielding. We therefore pursued another route, shown in Scheme 9, starting with L-xylose. This route has proved to be high yielding and reproducible, although the



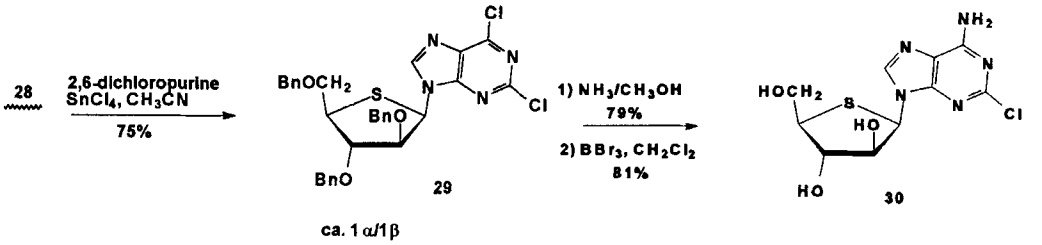
Scheme 7



Scheme 8



Scheme 9



Scheme 10

starting material is somewhat expensive. The use of **28** for the synthesis of a 4'-thioarabinonucleoside is shown in Scheme 10. It is noteworthy that a *ca.* 1:1 anomeric mixture was obtained in the *arabino* case with a purine base, in sharp contrast to the 2'-deoxy *ribo* series noted earlier, where the α anomer was strongly favored. Separation of the anomers is again best accomplished at the blocked nucleoside stage.

Biological Results

Cytotoxicity data on some of the compounds mentioned above is compiled in Table 1. It is clear that the 2'-deoxy compounds with thymine and 2-chloroadenine as the bases are quite cytotoxic. The *ribo* compound with a 2-chloroadenine base is significantly less cytotoxic. In previous work, we have found that the *ribo* compounds, even when they have significant cytotoxicity, have no *in vivo* selectivity for cancer cells, though of course that question will be answered for this series as well. In the P388 mouse leukemia model, 4'-thiothymidine (S-dT) at the maximum tolerated dose, 4 mg/kg/dose, on a qd1x5 (ip) schedule showed a 45% increase in lifespan.[18] This increase amounts to only modest activity, with no net change in tumor cell burden. That some selectivity was seen, however, is clearly a promising result in terms of other 2'-deoxy-4'-thionucleosides.

The significant cytotoxicity of S-dT, together with its modest selectivity in an animal tumor model, provided the impetus for a more in-depth examination of the mechanism of action of this compound.[19] Some of the data collected and conclusions drawn are summarized in this final section.

Table 1. Cytotoxicity Data: $IC_{50}(\mu M)$

Compound	H.Ep.2 (epidermoid)	CCRF-CEM (leukemia)	ACHN (renal)	SK-MEC-28 (melanoma)	H23 (lung)	SNB-7 (CNS)	CAKI-1 (renal)	DLD-1 (colon)
4'-Thiothymidine	0.06	0.66	12	0.15	0.3	0.12	-[b]	-
2'-Deoxy-4'-thiouridine	1.5	>4	-	-	-	-	-	-
2'-Deoxy-4'-thiocytodine	0.20	4	-	-	-	-	-	-
α-2'-Deoxy-4'-thiouridene	60	I[a]	-	-	-	-	-	-
2'-Deoxy-4'-thio-5,6-dihydro-5-azacytidine	>80	80	-	-	-	-	-	-
2-Chloro-2'-deoxy-4'-thioadenosine	<0.17	<1.7	1	1.6	-	-	0.30	66
2-Chloro-4'-thioadenosine	6	10	60	60	-	-	130	95
1-(2-Deoxy-4-thio-α-L-glycero-pento-furanosyl) thymine	6	12	-	4	1	14	40	7
1-(2-Deoxy-4-thio-β-L-glycero-pento-furanosyl) thymine	>150	>150	-	-	-	-	-	-
9-(2-Deoxy-4-thio-α-L-glycero-pento-furanosyl)-2-chloroadenosine	>130	>130	-	-	-	-	-	-

[a]No cytotoxicity at 160μM, the highest level tested; [b]Not tested

The metabolism of S-dT was examined in L1210 cells. Incubation with tritium-labeled S-dT resulted in the gradual appearance of one major metabolite identified as the triphosphate S-dTTP, with only traces of the mono- and diphosphate being seen. Thus, initial activation appears to be the rate-limiting step, with cellular kinases readily converting the monophosphate to the triphosphate.

In order to establish what enzyme was carrying out the initial phosphorylation of S-dT, its cytotoxicity was determined toward wild type CEM cells and CEM cells deficient in thymidine kinase (TK). The IC50 for the wild type line was 0.45 μM, while for the TK-deficient line it was 45 μM. This hundred-fold difference clearly indicates, as expected, that TK is the activating enzyme, and the modest inhibition seen in the deficient line probably results from the residual TK present in that cell line. Using the L1210 TK, kinetic parameters for S-dT as compared to thymidine (dT) were determined. For dT, the K_m was 1.2 μM and the V_{max} was 442 pmoles/min/mg, while for S-dT, the K_m was found to be 11 μM and the V_{max} 524 pmoles/min/mg. Thus the K_m of S-dT was about tenfold higher than for dT, with a comparable V_{max}.

Labeled S-dT was readily incorporated into L1210 DNA, indicating that the DNA polymerases responsible for most DNA synthesis in these cells efficiently utilize S-dTTP. There was a direct correlation between the inhibition of L1210 cell growth by S-dT and its incorporation into DNA. Incubation of cells for six hours with 0.2 μM S-dT resulted in incorporation of 50% of the S-dT into DNA, and essentially all of the material was eventually incorporated. Degradation of the DNA and reisolation of the labeled nucleoside demonstrated that the incorporated compound was S-dT and not some altered species. The S-dT that was incorporated into DNA was not removed for up to 72 hours, indicating that repair enzymes do not readily recognize the fraudulent nucleoside. In order to examine the effects of S-dT incorporation on the DNA produced, DNA polymerase α isolated from human cells was incubated with a labeled primer annealed to a 47-base template along with dATP, dGTP, dCTP, and either dTTP or S-dTTP. Examination of the DNA products produced by gel electrophoresis showed very similar patterns in both cases, suggesting not only that S-dT gets into the DNA efficiently, but also that it has little or no detrimental effect on further elongation after incorporation.

A number of nucleoside analogs have pronounced effects on nucleotide pool sizes, and dT itself is cytotoxic based upon its effects on the deoxycytidine nucleotide pools caused by feedback inhibition of ribonucleotide reductase. An examination of the effects of dT and S-dT on pools in L1210 cells was therefore undertaken. The expected reductions in the dCTP pool upon incubation of cells with 1, 10, or 100 μM dT were seen at 4 and 24 hours, with no effects on other dNTP pools. With S-dT, however, no reductions were seen with any of the dNTP pools; on the contrary, an increase in the dCTP pool was seen. In a further experiment, it was found that S-dT increased the metabolism of labeled 2'-deoxycytidine into both dCTP and dTTP, suggesting that the compound or a metabolite may have an effect on deoxycytidine kinase activity.

The above results suggest that S-dT has little effect on the pathways leading to nucleic acid synthesis, but rather exerts its cytotoxic effects after incorporation into DNA. As a further support to that hypothesis, that hypothesis, in another laboratory, S-dT has been incorporated into a dodecamer containing the *Eco*RV restriction endonuclease recognition site.[20] That incorporation has little effect on the melting temperature or the structure of the DNA. When the S-dT is incorporated at the cleavage site, however, the oligomer was not a substrate for the restriction enzyme, and was not recognized by the associated methylase.[20]

Summary

The pursuit of new 4'-thionucleosides has proven to be an interesting and challenging direction. Certain of the compounds have already been found to have biological activities suggestive of the potential for drug development. In the case of 4'-thionucleosides directed toward anticancer applications, it is clear that further investigation is warranted. One of the initial compounds produced, 4'-thiothymidine, has shown evidence of considerable cytotoxicity exerted by a mechanism distinct from other nucleoside anticancer agents.

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