erated were extracted into dichloromethane (2 × 10 mL). The combined dichloromethane extracts were dried (K_2CO_3) and TLC analyses showed only a single spot in all cases. The GLPC analyses of these amines showed single peak for amphetamine-d_0 (7.8 min), amphetamine-d_{11} (7.3 min), amphetamine-d_7 (7.6 min), and amphetamine-d_6 (7.3 min). Trifluoroacetic anhydride (0.5 mL) was added to each of the dried dichloromethane solutions and the mixture was left at room temperature overnight (12 h). The solvent, excess anhydride, and trifluoroacetic acid formed were then evaporated under reduced pressure. The trifluoroacetamide obtained was recrystallized from petroleum ether to give TFA–amphetamine-d_0 (mp 58–59 °C), TFA–amphetamine-d_{11} (mp 61 °C), TFA–amphetamine-d_7 (mp 57–58 °C), and TFA–amphetamine-d_6 (mp 58.5–59 °C). GLPC analyses of these TFA derivatives under the same condition as that of free amines gave retention times of 14, 13.5, 13.6, and 13.6 min for TFA derivatives of amphetamine-d_0, -d_{11}, -d_7, and -d_6, respectively.

Pharmacology. The spontaneous locomotor activity of Swiss–Webster albino mice ranging in weight from 22 to 24 g and housed in three circular, six-beam, photocell activity cages (Actophotometer, Metro-Scientific, Inc., Farmingdale, N.Y.) was determined. After the mice were received from the supplier (Scientific Small Animals, Arlington Heights, Ill.) they were acclimated to their new environment for at least 2 weeks. The animals were allowed free access to food and water until placed in activity cages. Two days prior to the experiment, the mice were randomly divided into groups of five mice each, and each mouse was used only once in an experiment. The testing was done between 11:00 a.m. and 5:00 p.m. daily. The amphetamine analogues (27.2 μmol/kg, i.e., 10 mg/kg for amphetamine-d_0, 10.6 mg/kg for amphetamine-d_{11}) as the sulfate salts were dissolved in normal saline and administered intraperitoneally in a volume of 0.2 mL/10 g of body weight. The control groups received a similar volume of normal saline and their spontaneous locomotor activity was determined daily.

Groups of five mice were treated and placed randomly in the activity cages. The number of interruptions of the light beam by the mice was recorded every 10 min in the first hour and then every hour for a period of 5 h. At least three groups of mice were used to determine the spontaneous locomotor activity of each amphetamine analogue. The results of these tests are shown in Table I.

Four analogues of amphetamine, namely, protioamphetamine (S), amphetamine-d_{11} (1), amphetamine-d_7 (2), and amphetamine-d_6 (3), were subjected to toxicity studies, using Swiss–Webster male mice weighing approximately 22 g. The drugs were injected intraperitoneally and the animals were watched for 24 h. Three doses (i.e., 313, 353, and 394 μM/kg) were used in determining the LD_{50} for each compound using 14–22 mice for each dose. Dose–response curves were drawn by linear regression analysis. LD_{50} (mg/kg) were obtained using the method of Litchfield and Wilcoxon.[15]

Acknowledgment. The authors are most grateful to Dr. Hemendra Bhargava for statistical treatment of the data and to Ms. Patricia A. Szczepanik of Argonne National Laboratory for the chemical-ionization mass spectral analysis.

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β-D-Arabinofuran[1′,2′:4,5]oxazolo-1,3,5-triazine-5-*N*-methyl-4,6-dione and Analogues, Unusually Specific Immunosuppressive Agents

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Sequential treatment of the protected β-D-arabinofuran[1′,2′:4,5]-2-aminooxazoline (2) with methyl isocyanate and diimidazole carbonyl afforded the 2,2′-anhydro-β-D-arabinofuranosyl nucleoside, **6**. Deprotection and hydrolysis yielded the corresponding arabinoside. Although the anhydronucleoside exhibited in vitro antiviral activity against herpes simplex type 1, it exacerbated the infection in vivo. Further examination uncovered an in vitro inhibition of the induction of a cell-mediated immune response without cytotoxicity.

As part of a program in our laboratory of examining the chemistry and pharmacology of the antiviral, antibiotic nucleoside, dihydro-5-azathymidine (1),[1] we have explored the syntheses of related structural types to expand its spectrum of biological activity. As an example of this, we report that the related 6-oxo arabinoside, **8a**, and the

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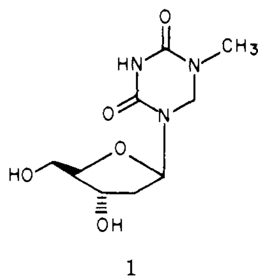


1

2,2'-anhydronucleoside, **7a**, exhibit immunosuppressive activity in contrast to 1. Although immunosuppression has been observed, and clinically exploited, with nucleosides in the past,[2] most of these attenuate the immune response by affecting various phases of both the humoral and cell-mediated immune (CMI) responses. However, the immunosuppressive activity of 7 and 8 appears unique in that it specifically affects only the induction of the CMI response, without causing nonspecific cytotoxicity.

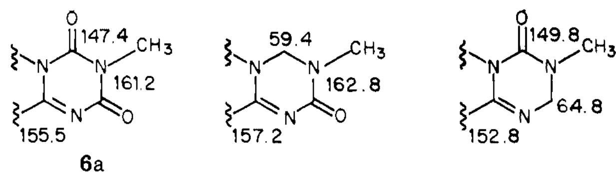
Chemistry. Although relatively few examples of 1,3,5-triazine nucleosides have been examined in depth, 5-azacytidine[3a,d] is a well-known exception because of its interesting bioactivity.[3b,c] The corresponding 6-oxo ribonucleoside analogue of **8a** has also been prepared[4] by classical condensation procedures, although no biological activity was reported.

Since anhydronucleosides have previously been utilized as synthons for such nucleoside analogues as ribo-, arabino-, and deoxynucleosides,[5] and the aminooxazoline of arabinose is readily available,[6] we examined the chemistry of **2** in preparing anhydro-1,3,5-triazine nucleosides.

The necessary prerequisite of 3',5'-diol protection is readily attained through the trimethylsilyl[7] or the more versatile *tert*-butyldimethylsilyl groups[1a] (Scheme I). Treatment of the monosodium salt of **2a** in tetrahydrofuran (THF) with 1 equiv of methyl isocyanate afforded only one isomer, **3**, by NMR and TLC. This is in contrast to the reaction under the same conditions with methyl isothiocyanate which yields a 1:1 mixture of isomers, **4** and **5**.[1a] Although the alternate isomeric structure analogous to **4** for the methyl isocyanate reaction cannot be definitely ruled out, comparison of its spectral data to that of **4** and **5**, whose structures have been unambiguously established,[1a] suggests **3** as the correct representation.

The ^{1}H NMR spectrum of the unsubstituted **2a** exhibited a doublet for $H_{1'}$ at 5.88 ppm. Analogously, **3** and **5** showed resonances at 5.95 and 6.00 ppm, while the $H_{1'}$ of the ring-N-substituted **4** was at 6.93 ppm. Similarly, the now ring-N-substituted triazine **6**, prepared from **3** by ring closure with 1,1'-diimidazole carbonyl, exhibited in $H_{1'}$ resonance at 6.41 ppm.

The assignment of the sp^2 carbon ^{13}C NMR resonances of **6a** was based on the spectra of the analogous deoxy-

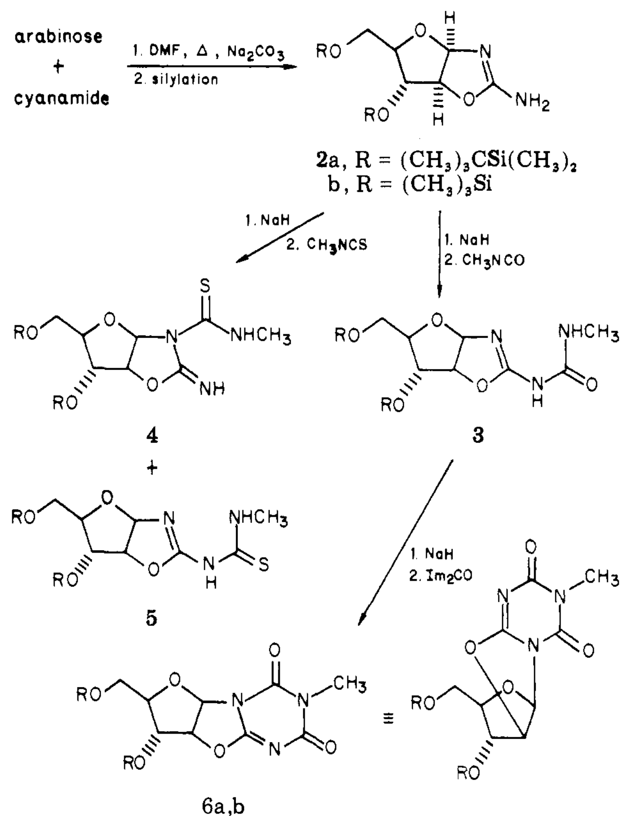


6a

S-triazine isomers[1a] as indicated below.

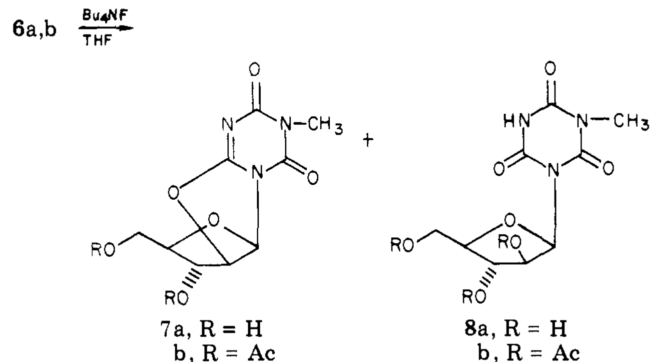
The divergence in isocyanate/isothiocyanate reactivity with ambident nucleophiles has been examined in an S vs. N system.[8] Similarly, the unpredictability of the reaction site on unsymmetrical, ambident nucleophiles with several different isothiocyanates has been reported.[9] The difference in regio- vs. nonregioselective reaction that we have observed appears to emphasize the need for a case by case

Scheme I



examination with caution in formulating predictive rules.

The sequence **2a** to **6a** was most easily accomplished, in greater than 80% isolated yield, by the sequential addition of base, methyl isocyanate, and 1,1'-diimidazole carbonyl in a "one-pot" reaction. The successful removal of the *tert*-butyldimethylsilyl protecting groups (**6a** to **7a**)



proved to be contingent upon the dryness, age, or mode of preparation of tetra-*n*-butylammonium fluoride. Our cursory observations indicated that freshly prepared tetra-*n*-butylammonium fluoride, particularly by ion exchange with the corresponding bromide,[10] afforded **7a**, whereas aged material (stored over 4A sieves in dry THF for several weeks) or material prepared from the corresponding hydroxide (with HF) yielded a mixture of **7a** and **8a**.

To secure larger quantities of **7a**, a more efficient approach was developed from the trimethylsilylaminooxazoline **2b**. Employment of *n*-butyllithium at low temperatures, followed by methyl isocyanate, 1,1'-diimidazole carbonyl, and an acidic workup, afforded **7a** in 33% yield after chromatography. Alternatively, ethyl acetate–methanol trituration of the crude material could replace the chromatography, giving a slightly lower yield. This procedure represents a three-step synthesis of the

Table I. Effect of Test Compounds on Mouse T-Effector Cell Function in an Alloantigen-Directed Cell-Mediated Cytolysis Assay[a]

	Compd concn, μg/mL					
	100		10		1	
Compd	% specific lysis ± SD	% of control[b]	% specific lysis ± SD	% of control	% specific lysis ± SD	% of control
7a	40 ± 3.2	92[c]	38.8 ± 2.7	88	43.4 ± 4.2	100
7b	40.3 ± 3.4	93	44.7 ± 2.5	103	41.1 ± 1.1	95
8a	40.3 ± 4.2	93	42.4 ± 3.5	98	40.8 ± 3.2	94
8b	38.3 ± 2.9	88	41.8 ± 3.2	96	41.8 ± 2.3	96

[a] The alloantigen specific cytolytic capacities of C57Bl/6 mouse spleen cells (H-2^b), sensitized 10 days previously with 50 × 10^6 DBA/2 mouse mastocytoma cells (H-2^d), are measured by a ^{51}Cr release assay. Sensitized spleen cells and ^{51}Cr-P815 labeled target cells (50:1 ratio) are incubated together for 4 h at 37 °C in RPMI 1640 media with Hepes buffer and 10% heat-inactivated calf serum. Test compounds are added to give the concentrations per milliliter above. All assays are performed in quadruplicate. After incubation of the mixture, the cells are pelleted by centrifugation and the supernatant is removed and counted for content of ^{51}Cr released from the target cells. The percent specific lysis is determined by the following: % specific lysis = [cpm (test supernatant) − cpm (spontaneous release)]/[cpm (total water lysis) − cpm (spontaneous release)] × 100. [b] The percent specific lysis by sensitized lymphocytes in the absence of test compound was 43.3 ± 2.7. [c] Compound 1 was tested in this system in a separate experiment, in which the control specific lysis was 49.5%. Compound 1 showed values of 73, 88, and 90% of control for 100, 10, and 1 μg/mL, respectively.

Table II. Effect of Test Compounds on the Ability of Mouse Spleen Cells to Generate a Cell-Mediated Immune Response in Vitro[a]

Treatment group	No. of cultures	Total no. of cells × 10^6 harvested	Mean no. × 10^6 cells/culture	Ratio of lymphocyte to target cell giving 50% specific lysis	No. of lymphocytes × 10^3/lytic unit[b]	No. of lytic units per culture
Control	12	30.0	2.5	6.0	150.0	16.7
1	18	39.2	2.2	7.2	178.8	12.2
7a	18	25.2	1.4	33.0	825.0	1.7
7b	18	50.0	2.8	8.5	211.3	13.2
8a	18	36.0	2.0	30.0	750.0	2.7
8b	18	41.6	3.2	10.5	262.5	12.2

[a] Normal C57Bl/6 mouse spleen cells (10 × 10^6/culture) were incubated for 5 days at 37 °C in RPMI 1640 media supplemented with 20% fetal calf serum and antibiotic in the presence of 0.5 × 10^6 $B_6D_2F_1$ mouse spleen cells. Cultures also contained 100 μg/mL/culture of test compound. After 5 days of culture the cells from identical cultures were pooled and their ability to lyse ^{51}Cr labeled P815-DBA/2 mouse mastocytoma cells was tested. Various culture cell to target cell ratios (minimum of three replicates/ratio) were mixed and incubated for 3 h at 37 °C in RPMI 1640 media buffered with Hepes and containing 10% heat-inactivated calf serum. Supernatants were collected and the ^{51}Cr released was counted. Percent specific lysis was calculated for each ratio and plotted against cell ratio. This plot was used to determine the number of cultured lymphocytes in one lytic unit. [b] One lytic unit in this experiment is defined as the number of lymphocytes required to give 50% specific lysis of 25 × 10^3 ^{51}Cr-P815 cells after 3 h of incubation.

anhydronucleoside **7a** from D-arabinose in greater than 20% yield overall.

In Vitro/in Vivo Antiviral Activity. In rabbit kidney monolayer cells inoculated with herpes virus type 1 (HSV-1),[11] the virus titer was diminished by 10–12-fold (log 6.1–4.9 PFU/0.5 mL) at a concentration of 25 μg/mL of dihydro-5-azathymidine (**1**). Similarly, the 2,2′-anhydronucleoside, **7b**, was essentially equipotent in this in vitro assay, although the arabinosides, **8**, exhibited no activity. However, the in vivo model of HSV-1 infection of mice indicated that **7a** exacerbated the infection. This was evident by comparison with **1** wherein SQ administration of **7a** at 150 mg/kg three times each day (days 0–4) showed a mean survival time (MST) of 5.4 days in comparison to control (vehicle) with an MST of 8.3 days and **1** (200 mg/kg) with an MST of 18.0 days.

In Vitro Immunological Profile. Since the antiviral studies suggested a possible in vivo immunosuppressive effect of **7a**, we examined the ability of **7** and **8** to either modulate the function of immunoreactive cells or to alter the induction of an immune response.

The ability of these compounds to alter the function of alloantigen-directed T-effector lymphocytes was examined using the ^{51}Cr release method, a measure of cell-mediated immunity. Results of this testing are shown in Table I. None of the compounds at any concentration tested showed any significant effect on the ability of spleen cells from alloantigen-sensitized mice to lyse specific target cells. All values were in the range of 90–100% of control values. In a separate experiment **1** showed an inhibition in this system of 27, 12, and 10% (at 100, 10, and 1 μg/mL, respectively). However, control values for nonspecific compound effects and normal spleen cells on target cells indicated that inhibition was due to direct compound cytotoxicity and not due to functional inhibition.

For further immunological characterization, **1**, **7a,b**, and **8a,b** were examined for their ability to alter the in vitro induction of a cell-mediated immune response. This experiment was designed to look at the afferent limb of a cell-mediated immune response in contrast to the efferent limb of the response examined in the previous experiment.

Cultures of C57Bl/6 and B_6D_2 mouse spleen cells were incubated for 5 days in the presence of 100 μg of test compound per milliliter per culture. At the end of the 5 days of incubation, cultures were assayed for the amount of T-effector lymphocyte function present by using the ^{51}Cr release system. Using various recovered, cultured lymphocyte to target cell (P815) ratios, the number of lytic units of activity/culture can be determined and represents a quantitative measure of the ability of an unknown compound to alter the inductive phase of cell-mediated immune response. In this experiment a lytic unit was defined as the number of cultured cells required to give 50% lysis of 25 × 10^3 target cells (^{51}Cr-P815) after 3 h of incubation at 37 °C.

The results of this experiment are shown in Table II. Compound **1** and the acetyl esters of **7** and **8** showed no

significant effect on the ability to generate a cell-mediated immune response in vitro. The addition of **7a** and **8a** to the culture system resulted in 90 and 84% inhibition of the response, respectively. In both cases the number of cells recovered per culture was less than control values (1.4 and 2.0×10^6 vs. 2.5×10^6). However, this reduction (a measure of direct cytotoxicity) was insignificant and not enough to account for the nearly tenfold decrease in recoverable lytic activity associated with compounds **7a** and **8a**. In a separate experiment the test compounds were added to the culture system on day 4 of the 5-day response. Events late in the culture system such as differentiation and proliferation to amplify the response were unaltered by any of the test compounds. Recoverable lytic units were 90–100% of control values.

Since only the induction of the cell-mediated immune response and not the function of sensitized T-effector lymphocytes was effected by compounds **7** and **8a**, another culture immune response induction system was utilized to determine the selectivity, if any, of these compounds. The in vitro induction of a humoral immune response as measured by the number of IgM-anti-sheep erythrocyte antibody plaque-forming cells (PFC) was used. The results (Table III) show that none of the compounds tested in this way significantly alters the number of PFC generated in 5 days of culture.

Summary

The synthesis of anhydro- and arabino-*S*-triazine nucleosides is readily attained by elaboration of a protected arabinoaminooxazoline. These nucleosides exhibit unique immunological properties in that they, in contrast to dihydro-5-azathymidine, exhibit a noncytotoxic inhibitory effect on the induction of a CMI response with no direct effect on T-effector cells or the induction of the humoral immune response. The inhibition of the induction of a CMI response may be due to effects on antigen recognition, differentiation, or processing events. These data suggest that an in vivo application on the compounds' selective immunosuppressive effects would necessitate drug administration close to the time of antigenic insult.

Experimental Section

All solvents employed were reagent grade. Tetrahydrofuran was distilled from sodium–benzophenone under N_2 prior to use. All reagents were used as received and moisture-sensitive materials were stored over indicating calcium sulfate.

^{1}H NMR spectra were recorded on either a Varian A-60A or T-60 in $CDCl_3$ with internal Me_4Si unless otherwise noted. GC–MS data were recorded on either an LKB 9000 or Varian Mat CH-7. ^{13}C NMR spectra were recorded on a Varian CFT-20 with all values referenced to internal Me_4Si. GC was performed on a Hewlett-Packard 402 with glass columns and He carrier gas.

3′,5′-Bis(*o-tert*-butyldimethylsilyl)-β-D-arabinofuran[1′,2′:4,5]oxazolo-1,3,5-triazine-5-*N*-methyl-4,6-dione (6a). To 51 mg (1 mmol) of 45% NaH (washed twice with hexane to remove oil) in 7 mL of dry THF (distilled prior to use from Na–benzophenone) under N_2 at room temperature was added 402 mg (1 mmol) of aminooxazoline I in several portions. After 30 min of stirring 75 μL (1.25 mmol) of methyl isocyanate was added dropwise over 5–10 min. After being stirred for 1 h the solution was either worked up or 200 mg (1.2 mmol) of diimidazole carbonyl was added and stirred for another 2 h. If the intermediate was desired, the workup procedure (vide infra) yielded 445 mg (97% yield) of a white solid which appeared by TLC and NMR to be one isomer: IR 3350, 3500 (NH) ($CHCl_3$), 1660 (C=O), 1520 cm^{-1} (amide II band); NMR ($CDCl_3$) δ 6.4 (1 H, br, NH), 5.95 (1 H, d, J = 5.5 Hz, C-1′), 4.78 (1 H, d, J = 5.5 Hz, C-2′), 2.85 (3 H, d, J = 5 Hz, NCH_3).

If the reaction was continued by addition of the diimidazole carbonyl, the workup included taking the heterogeneous solution up in 100 mL of EtOAc and 50 mL of water and washing the organic layer with 50 mL of brine. The aqueous solution was back-extracted with EtOAc which was then combined with the original organic phase after washing with brine and drying over Na_2SO_4. Concentration under reduced pressure yielded an oil which was chromatographed on 150 g of silica gel with 40% EtOAc–hexane to afford ultimately 414 mg (85% yield): IR 1760, 1700, 1660 cm^{-1} (C=O, C=N), (CCl_4); NMR ($CDCl_3$) δ 6.41 (1 H, d, J = 5.5 Hz, C-1′), 5.18 (1 H, d of d, J = 5.5 Hz, C-2′), 3.32 (3 H, 5, NCH_3), 0.90 and 0.83 (18 H, s, *t*-Bu); UV (EtOH) λ_{max} 217 nm (ε 5250), λ_{min} 275 nm (ε 250); TLC R_f 0.63, final product, single spot (50% EtOAc–hexane), R_f 0.48, intermediate; GLC retention time = 8.9 min, single peak with full-scale deflection (6-ft 3.8% UCW-98, 270 °C); GC–MS 485 (<1, M$^+$), 470 (3, M$^+$ – Me), 428 (62, M$^+$ – *t*-Bu), 282 (13), 261 (29), 189 (100).

3′,5′-Bis(*O*-acetyl)-β-D-arabinofuran[1′,2′:4,5]oxazolo-1,3,5-triazine-5-*N*-methyl-4,6-dione (7b). To 2.5 g (5.15 mmol) of **6a** in 50 mL of dry THF under N_2 at room temperature with stirring was added 15.5 mL of a 1 M tetra-*n*-butylammonium fluoride solution. After 2 h the solution was concentrated in vacuo and the resultant gum was taken up in 20 mL of pyridine to which was added 5 mL of acetic anhydride and stirred under N_2 at room temperature for 4 h. Upon concentrating the solution was applied to a 150-g silica gel column prepared in EtOAc and after an initial 200-mL elution, the solvent was changed to 5% MeOH–EtOAc. Recovered was 1.2 g (70%) of a white solid (after trituration with ether): NMR (Me_2SO-d_6) δ 6.38 (1 H, d, J = 5.5 Hz, C-1′), 5.55 (1 H, d, J = 5.5 Hz, C-2′), 5.33 (1 H, m, C-3′), 4.53 (1 H, m, C-4′), 4.1 (2 H, m, C-5′), 3.15 (3 H, s, NCH_3), 2.11 and 1.95 (6 H, s, CH_3CO); TLC R_f 0.57, single spot (5% MeOH–EtOAc); GLC retention time = 4.8 min, single peak with full-scale deflection (240 °C, 4-ft 3.8% UCW-98); GC–MS 341 (10, M$^+$), 298 (3, M$^+$ – CH_oCO), 239 (12, M$^+$ CH_3 – $2CH_3CO$ – H), 151 (75), 43 (100).

2′,3′,5′-Tri-*O*-acetyl-β-D-arabinofuranosyl-1,3,5-triazine-5-*N*-methyl-2,4,6-trione (8b). The procedure to prepare **7b** was employed on 15.5 g (32 mmol) of **6a** with appropriate scale-up of reagents except that the tetra-*n*-butylammonium fluoride–THF solution was aged. Also, twice the usual 50% excess of reagent was needed to effect complete desilylation (by TLC). Proceeding with the remainder of the procedure and chromatography resulted in 9.16 g (72%) of a white solid (mp 160–161 °C, 5% EtOAc–hexane recrystallized): NMR ($CDCl_3$) δ 7.6 (1 H, br s, NH), 6.70 (1 H, d, J = 8 Hz, C-1′), 5.5–6.0 (2 H, m, C-2′, C-3′), 4.0–4.5 (3 H, m, C-4′,5′), 3.30 (3 H, s, NCH_3), 1.93, 1.90, 1.87 (9 H, s, CH_3CO); TLC R_f 0.68 (5% MeOH–EtOAc); GLC retention time = 6.8 min (240 °C, 4-ft 3.8% UCW-98); GC–MS no M$^+$ 401, 343 (1, M$^+$ – CH_3CO – CH_3), 342 and 341 [2, M$^+$ – CH_3CO_2(H)], 328 (9, M$^+$ – CH_3CO_2 – CH_3), 249 [16, M$^+$ – CH_3CO_2H – 2(CH_3CO) – CH_3], 43 (100, CH_3CO). Anal. ($C_{15}H_{19}N_3O_{10}$) H, N; C, calcd, 44.89; found, 45.48.

β-D-Arabinofuran[1′,2′:4,5]oxazolo-1,3,5-triazine-5-*N*-methyl-4,6-dione (7a) from 2b. To 22.26 g (70 mmol) of **2b** in 300 mL of dry THF at –78 °C under N_2 was added 47 mL of *n*-butyllithium (ca. 1.6 M in hexane). After the addition was complete (15 min), the cold bath was removed and 5.25 mL of methyl isocyanate was added at a reaction temperature of 0 °C. After 5 min 12.5 g (77 mmol) of 1,1′-diimidazole carbonyl was added and the reaction stirred for 1 h while warming to room temperature. The solution was taken up in ethyl acetate–H_2O (2:1 L), shaken, and separated. The aqueous phase was rewashed with 1 L of ethyl acetate; the organic phases were combined, dried over sodium sulfate, and concentrated in vacuo. To the residue was added 300 mL of methanol, 30 mL of water, and 5 mL of acetic acid. The resulting solution was concentrated at reduced pressure at less than 40 °C, followed by azeotroping under the same conditions with 600 mL of methanol followed by 600 mL of acetonitrile. The resulting gum was chromatographed on 400 g of silica gel with 5% methanol–ethyl acetate to 10% to yield 5.93 g of a white solid (33% yield). Recrystallization from 25% methanol–ethyl acetate gave mp 246–248 °C. Alternatively, the product could have been isolated by trituration of the crude mixture with 20% methanol–ethyl acetate followed by filtration. The solid was washed once with the same solvent to remove the last traces of imidazole: NMR (D_2O) δ 6.59 (1 H, d, J = 5.5 Hz, C-1′), 5.50 (1 H, d, J = 5.5 Hz, C-2′), 4.7–4.3 (2 H, m, C-3′,4′), 3.64 (2 H, d, J = 3.5 Hz, C-5′), 3.23 (3 H, s, NCH_3). Anal. ($C_9H_{11}N_3O_6$) C, H, N.

Table III. Effect of Test Compounds on the Ability of Mouse Spleen Cells to Generate a Humoral Immune Response to Sheep Erythrocytes in Vitro[a]

Treatment group	Mean no. of plaque-forming cells[b]/culture ± SD			
	Vehicle	100 μg/culture	10 μg/culture	1 μg/culture
Control	6440 ± 2266			
7a		7107 ± 2428	4853 ± 1840	4227 ± 829
7b		8720 ± 2724	5933 ± 1564	5240 ± 1391
8a		6293 ± 958	5520 ± 592	6240 ± 1254
8b		10040 ± 4811	4307 ± 1310	4907 ± 1567

[a] Normal C57B1/6 mouse spleen cells (10×10^6/culture) were incubated for 5 days at 37 °C in RPMI 1640 media supplemented with 20% fetal calf serum and antibiotic in the presence of 10^7 sheep erythrocytes (SRBC). Compound was added to the cultures (three cultures per concentration) to give the final concentration/culture shown above. [b] After 5 days of culture the cells were harvested and washed, and the number of cells producing IgM-anti-SRBC antibody was enumerated by the hemolytic plaque-forming cell assay. See Methods.

β-D-Arabinofuran[1',2':4,5]oxazolo-1,3,5-triazine-5-*N*-methyl-4,6-dione (7a) from 7b. To 50 mg (0.15 mmol) of **7b** was added under N_2 at room temperature with stirring 2 mL of ammonia-saturated methanol. After 30 min the solution was concentrated under reduced pressure and the residue washed twice with chloroform, taken up in water, and lyophilized to yield 35 mg of a white solid (93%), which by TLC (R_f 0.46; 30% MeOH–EtOAc) and NMR was identical with **7a** prepared from **2b**.

In Vitro Immunological Profiling. A. ^{51}Cr Release Assay (based on that described by Brunner et al.[12]). C57B1/6 (H-2^b) mice were sensitized to H-2^d alloantigens by the intraperitoneal (ip) injection of 30–50 × 10^6 P815 mastocytoma cells [ascites tumor derived and passaged in DBA/2 (H-2^d) mice]. Ten days after allosensitization, the spleens from five to seven animals were removed, teased into single cell suspension, pooled, washed by centrifugation, and counted (viability stain 0.1% trypan blue in saline). Spleen cells were mixed in 10 × 75 mm glass tubes with ^{51}Cr-labeled P815 cells (200 μCi of sodium chromate–^{51}Cr per 5 × 10^6 P815 cells incubated for 30 min at 37 °C and repeatedly washed) at a ratio of 50:1 in a volume of 0.25 mL of RPMI 1640 media with Hepes buffer (Gibco). Test compounds in a volume of 0.05 mL were added to the mixture. The mixture was incubated for 4 h at 37 °C in an atmosphere of 83% nitrogen, 10% carbon dioxide, and 7% oxygen. At the end of the incubation 0.7 mL of cold saline was added to each tube. The tubes were centrifuged, the supernatant (test) was collected and placed in a counting tube, and the amount of ^{51}Cr released was determined by counting in a Packard Auto-Gamma scintillation spectrometer. Controls for this system were compound incubated with normal spleen cells and ^{51}Cr-P815 cells (spontaneous release), ^{51}Cr-P815 cells incubated with compound (cytotoxicity control), and ^{51}Cr-P815 cells with water (total water lysis). The percent specific cytotoxicity (^{51}Cr release) was calculated as follows.

$$\%\ \text{specific cytotoxicity} = \frac{\text{cpm (test)} - \text{cpm (spontaneous release)}}{\text{cpm (water lysis control)} - \text{cpm (spontaneous release)}} \times 100$$

B. In Vitro Induction of a Cell-Mediated Immune Response. The method used was a modification of that described by Wunderlick and Canty.[13] C57B1/6 mice were sacrificed by cervical dislocation and their spleens aseptically removed, teased into single cell suspension, washed by centrifugation with media RPMI 1640, and counted for viability with 0.1% trypan blue. B_6D_2 (F_1) mouse spleen cells which serve as antigen were prepared the same way. Ten million C57B1/6 spleen cells and 0.5 × 10^6 B_6D_2 spleen cells were cocultivated in 1-mL total volume in the presence or absence of test compound (added in 0.1-mL volume) in 10 × 35 mm wells of Multidish plates (Linbro). A minimum of 12 individual cultures per compound concentration to be tested was used. The culture media was RPMI 1640 with 20% fetal calf serum (Gibco) and gentamycin (Schering). Multiple cultures for each test compound and a media control were incubated for 5 days at 37 °C in an atmosphere of 83% nitrogen, 10% carbon dioxide, and 7% oxygen. At the end of the culture period, the cells from all like cultures were pooled, washed, counted, and assayed by the ^{51}Cr release method for the amount of specific cytotoxicity generated, expressed as lytic units per culture. In these experiments a lytic unit was defined as the number of culture-generated effector cells necessary to give 50% lysis of 25 × 10^3 ^{51}Cr-P815 target cells after 3 h of incubation at 37 °C.

C. In Vitro Induction of a Humoral Immune Response. A modification[14] of the spleen cell culture system of Mishell and Dutton[15] was used. Spleen cells (10 × 10^6) from C57B1/6 mice were cultured with 10^7 sheep erythrocytes (SRC) in RPMI 1640 media supplemented with 20% fetal calf serum and gentamycin. Test compound was added to each 1-mL culture in a volume of 0.1 mL of RPMI 1640. Cultures receiving 0.1 mL of media without drug served as controls. Each compound concentration was tested in triplicate cultures. After 5 days of incubation at 37 °C in 83% nitrogen, 10% carbon dioxide, and 7% oxygen, the humoral immune response generated was quantitated by enumerating the IgM-anti-SRS antibody secreting plaque-forming cells (PFC) per culture by the PFC assay of Jerne and Nordin[16] as modified by Plotz et al.[17]

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