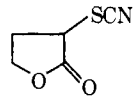
Table IV. Sample Calculation of Mean Substructure Activity Frequency (MSAF) for One of 489 Compounds Whose Probability of Antiarthritic–Immunoregulatory Activity Was Predicted



SK&F 43248 inactive

Substructure and description	Occurrences in first 770 (T)	Occurrences in actives among first 770 (A)	Frequency of act. (SAF = A/T)
H26, lactone	51	8	0.1568
IWB, saturated C_4O ring	68	18	0.2647
000, carbon isolated in a functional group	198	49	0.2474
862, Het-SCN	1	0	0.0000[a]
69/Y, one ring	549	126	0.2295
71/Y, isolated heterocycle	343	77	0.2244
71/5, five-membered heterocyclic ring	120	32	0.2666
72/4, one oxygen in ring	213	56	0.2629
72/7, only one heteroatom in ring	328	83	0.2530
74/1, substitution α to a heteroatom	468	117	0.2500
74/2, substitution β to a heteroatom	245	58	0.2367
74/4, 1,2 substitution	150	34	0.2266
77/6, X-C-C-C-Y	96	20	0.2083
78/Y, X-C-C=Y	113	22	0.1946
		13	3.0215
		MSAF =	0.2324

[a] The SAF value for substructure 862 is not included in the MSAF computation because of insufficient representation among the first 770 compounds. (See text.)

Table V. Occurrence of Antiarthritic–Immunoregulatory Activity among 489 Compounds Grouped According to Mean Substructure Activity Frequency (MSAF). MSAF Values are Calculated on the Basis of Data from 770 Previously Tested Compounds

MSAF range	No. tested	No. active[a]	Frequency of act.
>0.26	80	18	0.225
0.25–0.26	85	18	0.212
0.24–0.25	127	13	0.102
0.23–0.24	116	14	0.121
<0.23	81	11	0.136
Totals	489	74	

[a] The difference of these values from the random values (12.1, 12.9, 19.2, 17.6, and 13.0, respectively) is marginally significant ($p < 0.1$). If the compounds with MSAF >0.25 (top two MSAF ranges together) are compared to the compounds with MSAF <0.25 (bottom three MSAF ranges together), the difference between the observed numbers of actives (36 and 38) and the most probable or "random" values (25 and 49, respectively) is highly significant ($p < 0.01$).

ranges is shown in Table V. While there is no difference among the lower three MSAF ranges shown, activity is clearly and significantly ($p < 0.01$) less frequent among compounds in the lower three ranges when compared with the higher two MSAF ranges. Considering the limitations of the biological data used and the coarse discriminatory power of the available substructural system, we are encouraged by the results.

The application of this method to other substructural systems (such as those based on Wiswesser notation) and to other sets of biological data is clearly indicated. In the context of large screening programs this technique may be of practical value, even in its present primitive form, by improving the efficiency of "lead" generation.

Although alternative computational procedures can be explored, more useful correlations (*i.e.*, prediction of larger differential probabilities of activity) will probably require a more sophisticated substructural system based on direct computer manipulation of complete structural records.[7] Ultimately, perhaps in an evolutionary process guided by substructural analysis of many biological data, this system could place emphasis on those molecular features that prove to be of fundamental significance to biological mechanisms. Advances in computers and programming technology[8] are beginning to make feasible the systematic study of factors as complex as three-dimensional structure, polarizability, bonded and nonbonded interactions, and solvation phenomena.

Acknowledgment. We thank Dr. A. D. Bender for stimulating discussion and continuing encouragement.

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Preparation and Antitumor Activity of 4′-Thio Analogs of 2,2′-Anhydro-1-β-D-arabinofuranosylcytosine

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Among nucleosides with antitumor activity, 1-β-D-arabinofuranosylcytosine has well-known activity against rodent and human neoplasms.[1] The drug has been used clinically against acute leukemia and lymphoma.[2] Because the compound produces megaloblastosis and chromosomal alteration in bone marrow,[2a,3] we have prepared a sulfur analog with the hope that it might be less toxic. Hopefully, also, the analog might be less rapidly deaminated to inactive spongouridine.[4]

Since 2,2′-anhydro-1-β-D-arabinofuranosylcytosine is less easily deaminated[5] than the straight nucleoside, we have also prepared the 4′-thio analog of the anhydro nucleoside.

It is often observed that low yields of nucleosides are obtained by condensing 4-thio-D-ribofuranosyl derivatives with pyrimidine bases. However, the condensation of 1,2,3,5-tetra-*O*-acetyl-4-thio-D-ribofuranose and bis(trimethylsilyl)-*N*-acetylcytosine with stannic chloride as catalyst[6] gave a 65% yield of the acetylated β-D nucleoside *I* with the α-D nucleoside in 3% yield.

Table I. Inhibition of Growth of KB Cell by 4′-Thio-1-β-D-arabinofuranosylcytosine and Derivatives

	Concn (M) for 50% growth inhibition
2,2′-Anhydro-1-β-D-arabinosylcytosine hydrochloride	1.9×10^{-7}
1-β-D-Arabinosylcytosine	1.6×10^{-7}
2,2′-Anhydro-4′-thio-1-β-D-arabinosylcytosine hydrochloride	4.3×10^{-7}
4′-Thio-1-β-D-arabinosylcytosine	4.2×10^{-7}

Acetyl groups were removed from I by treatment in MeOH-NH_3 at 100° in a sealed tube. The nucleoside analog II obtained in 60% yield was identical with the nucleoside made earlier in our laboratory by the fusion of 4-thio-D-ribofuranosyl chloride with 2,4-diethoxypyrimidine.[7]

Cyclization between the 2 and 2′ positions of the 4′-thiocytidine was conducted[7] in the presence of phosphoryl chloride in DMF at 25°. Reaction products were separated on Amberlite IR-120 (pyridinium form) by stepwise elution using pyridine formate buffer (pH 4.8). 2,2′-Anhydro-4′-thio-1-β-D-arabinofuranosylcytosine (III) was isolated as the hydrochloride in 50% yield after passing the gummy formate derivative through Amberlite IR-400 (chloride form). The uv spectrum of this compound at pH 2.0 no longer showed maximum absorption at 282 nm, which is characteristic of 4′-thiocytidine hydrochloride. Instead, the first maximum absorption was at 268 nm (ε 10,800) and the second maximum was at 232 nm (ε 9800). The shift of absorption maximum evidences the loss of benzenoid conjugation in the base moiety.

In the nmr spectrum of compound III, the anomeric proton signal was a doublet at δ 6.70 with a coupling constant of 8.5 Hz while the signal at δ 5.90 with the same coupling constant can be assigned to the 2′ proton by the double resonance technique. Shift of the 2′ proton in compound III to lower field than that of compound II indicated that the cyclization reaction occurred between the 2 and 2′ position.

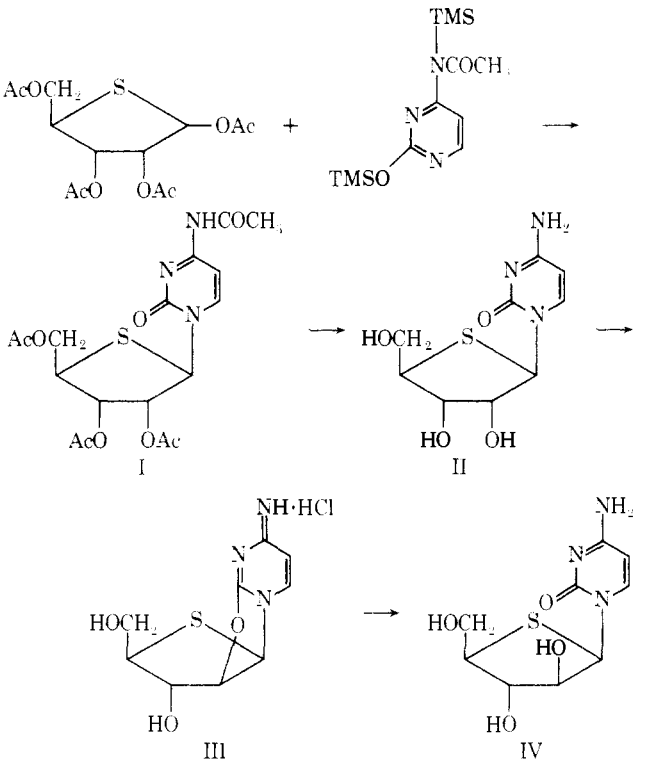
The large coupling constant of 8.5 for III compared to 6.0 for the 4′-oxygen compound cannot be caused by difference in dihedral angles because of lack of flexibility in the highly cyclized system and, hence, must result from the large difference in electronegativity of sulfur compared to oxygen.[8]

The ring opening of III by aqueous ammonia gave 4′-thio-1-β-D-arabinofuranosylcytosine (IV) in quantitative yield. The configuration of compound IV was thus established as β-D. The cytosine group in IV was recognized by its maximum absorption at 280 nm and the presence of the D-arabinofuranosyl moiety was recognized by its nmr spectrum, which showed a signal for the 3′ proton at 4.15 as a triplet with a coupling constant of 8.0 Hz.

The optical rotation of compound IV (+72.8°) was different from that of the now known 4′-thio-α-D-arabinofuranosylcytosine (+143°), previously prepared by the fusion of 4-thio-D-arabinofuranosyl chloride with bis(trimethylsilyl)-N-acetylcytosine, followed by deacetylation.[9] As previously discussed,[10] Hudson's isorotation rule applies to 4′-thio-D-ribofuranosyl pyrimidine nucleosides, although the rule is not obeyed in the corresponding 4′-oxygen analogs[11] (Scheme I).

The growth-inhibiting activities of 2,2′-anhydro-1-β-D-arabinofuranosylcytosine and 1-β-D-arabinofuranosylcytosine on KB cell were compared with the corresponding 4′-thio analogs III and IV. The concentrations of these drugs to effect a 50% reduction on cell numbers are summarized in Table I. Although the activities of the 4′-thio analogs are not greatly different from the corresponding 4′-oxygen

Scheme I



analogs in this preliminary test, the fact that the 4′-thio analogs have comparable high activities is of interest and indicates the value of further biochemical examination of the sulfur analogs especially for activity, persistance, and toxicity *in vivo*.

Experimental Section

2′,3′,5′-Tri-*O*-acetyl-4′-thio-1-β-D-ribofuranosyl-*N*-acetylcytosine (I). A mixture of 1,2,3,5-tetra-*O*-acetyl-4-thio-D-ribofuranose (3.34 g, 0.01 mol) and bis(trimethylsilyl)-*N*-acetylcytosine (3.57 g, 0.012 mol) in 1,2-dichloroethane was stirred for 20 hr at 25° in the presence of stannic chloride (3.64 g, 0.014 mol). After addition of saturated aqueous sodium bicarbonate solution (50 ml), the mixture was filtered over Celite and the filtrate was extracted by two 100-ml portions of chloroform. The chloroform layer was dried over sodium sulfate and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using chloroform–acetone (9:1) as a solvent to give two anomeric nucleosides. The faster moving compound on evaporation gave 130 mg (3%) of gummy 2′,3′,5′-tri-*O*-acetyl-4′-thio-β-D-ribofuranosyl-*N*-acetylcytosine: $[\alpha]^{25}D$ +12.0° (*c* 1.0, $CHCl_3$), uv_{max} (EtOH) 298 nm (ε 18,000), 248 (6700). *Anal.* ($C_{16}H_{21}N_3O_8S$) C, H, N, S. The slower moving blocked nucleoside I was isolated as a gum (2.78 g, 65%): $[\alpha]^{25}D$ −13.5° (c 1.0, $CHCl_3$); uv_{max} (EtOH) 296 nm (ε 18,000), 248 (6700); nmr ($CDCl_3$) δ 2-2.2 (4 $COCH_3$), 6.63 (1 H, d, J = 7.0 Hz), 7.83, 8.54 (AX type, J = 8.0 Hz). *Anal.* ($C_{16}H_{21}N_3O_8S$) C, H, N, S.

4′-Thio-β-D-ribofuranosylcytosine Hydrochloride (II). 2′,3′,5′-Tri-*O*-acetyl-4′-thio-1-β-D-ribofuranosyl-*N*-acetylcytosine (3.0 g, 6.6 mmol) was dissolved in 50 ml of neat methanol and the mixture was saturated at 0° with anhydrous ammonia and then heated in a sealed tube at 100° for 20 hr. The reaction solution was concentrated under reduced pressure to a yellow gum, which was dissolved in 95% ethanol, and several drops of concentrated hydrochloric acid were added. After cooling, the crystalline product was collected by filtration and recrystallized from 90% ethanol. The yield of compound II was 1.1 g (55%). The melting point was identical with that of the compound which was previously prepared in our laboratory:[12] $[\alpha]^{25}D$ −5.5° (*c* 3.0, water) [reported value −3.4° (*c* 3.2, water)].

2,2′-Anhydro-4′-thio-1-β-D-arabinofuranosylcytosine Hydrochloride (III). Phosphoryl chloride (6.0 g, 39 mmol) was placed in 20 ml of DMF and the mixture kept at 25° for 30 min. To the solution was added 1.0 g (3.4 mmol) of II and the mixture was stirred at 25° for 5 hr and then poured into 100 ml of cold water to

destroy the reagent. The aqueous reaction mixture was applied to a Amberlite IR-120 (pyridinium form) column (2.5 × 45 cm). The column was eluted with 0.1 *M* pyridine formate (pH 4.8) to give recovered material and subsequently with 0.4 *M* pyridine formate (pH 4.8) to afford the product. The fraction eluted by 0.4 *M* pyridine formate was evaporated to dryness after the pH of the solution was adjusted to 4.0 with formic acid. Repeated evaporation of the residue with ethanol gave a gum, which was dissolved in 5 ml of water. The solution was then passed through a column (2 × 4 cm) of Amberlite IR-400 (chloride form). The column was washed with 150 ml of water and the combined effluent and washing were evaporated to dryness under reduced pressure. Recrystallization of the residue from 90% ethanol afforded 50% of III hydrochloride: mp 241–243° dec: $[\alpha]^{25}D$ −167.7° (*c* 5.0, water); uv_{max} (water, pH 2.0) 268 nm (ε 10,800), 232 (9800): nmr (D_2O) δ 5.90 (1 H, br d, *J* = 8.5 Hz), 6.70 (1 H, d, *J* = 8.5 Hz), 6.85, 8.37 (AX type, *J* = 7.0 Hz). *Anal.* ($C_{19}ClH_{12}N_3O_3S$) C, H, N, S.

4′-Thio-1-β-D-arabinofuranosylcytosine (IV). III hydrochloride (100 mg) was dissolved in 3 ml of water and the solution was adjusted to pH 9 with aqueous ammonia. After 10 min at 25° the mixture was acidified with hydrochloric acid and applied to a column (1 × 2 cm) of Amberlite IR-120 (acid form). The column which was washed well with water was eluted with 100 ml of 1 *N* NH_4OH. The eluent was evaporated under reduced pressure and the residue crystallized from ethanol to produce 85 mg (90%) of pure IV: mp 221–222° dec; $[\alpha]^{25}D$ +72.8° (*c* 2.0, water); uv_{max} (water, pH 2.0) 280 nm (ε 12,100); nmr (D_2O) δ 2.48 (1 H, double t, *J* = 6.0, 8.0 Hz), 4.10 (2 H, d, *J* = 5.0 Hz), 4.15 (1 H, t, *J* = 8.0 Hz), 4.58 (1 H, double d, *J* = 6.0, 8.0 Hz), 6.41 (1 H, d, *J* = 6.0 Hz), 6.50, 8.78 (AX type, *J* = 8.0 Hz). *Anal.* ($C_9H_{13}N_3O_4S$) C, H, N, S.

Biological Assays. The *in vitro* antitumor assays were conducted according to the protocol cited in the Cancer Chemotherapy Report.[11] KB cells grown in monolayer cultivers were obtained from Flow Laboratories, Rockville, Md. The cells were grown in petri dishes (3 cm in diameter) with the minimum essential medium,[13,14] supplemented with 10% fetal calf serum and glutamine (2 m*M*). All tests were made during log phase. Aliquots (0.2 ml) of medium containing the various concentration of drugs were added to 2-ml portions of medium containing approximately 5 × 10^4 cells. 6-Mercaptopurine was used as a positive control. Incubation was conducted for 72 hr, after which time the cells were washed by Earl's balanced solution, trypsinized with EDTA, and counted in a Coulter counter. The base line of cell count was made after 24 hr from inoculation of cells. During a further 48 hr the cell number in the controls increased five- to sevenfold. The effective concentration of 6-mercaptopurine for 50% reduction of growth was shown to be 0.35 g/ml of medium.

Acknowledgment. This work was supported in part by Grant No. RO1 CA 12422 from the National Institute of Health.

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Hydroxyproline Analogs of Bradykinin†

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Alterations in the proline residues of the peptide hormone bradykinin (BK) (Figure 1) produce profound effects on the biological activities of the resulting analogs.

Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
1 2 3 4 5 6 7 8 9

Figure 1. The structure of bradykinin.

This was first observed by Schröder[2] who found that replacement of proline 3 with alanine caused no change in the biological activity while [Ala²]-BK and [Ala⁷]-BK had about 1% of the potency of BK. Later, Stewart[3] found that the effect on biological activity of D-proline in positions 2 or 7 paralleled that of alanine in those positions, while [D-Pro³]-BK had only 1/10,000 the potency of bradykinin. Circular dichroism (CD) spectra showed[4] that the solution structures of BK and its highly potent analogs were quite similar, while several analogs with very low biological activities had solution conformations very different from that of BK. However, no unique correlation between conformation and biological activity could be made, since several inactive analogs had CD spectra very similar to that of BK. Among the proline analogs, [D-Pro⁷]-BK had a spectrum very similar to that of BK, differing only in the intensity of that 220-nm peak, while that of [D-Pro²]-BK was very different, being inverted in this region.

From these data it would appear that with respect to position 7 of BK the ring of proline plays a significant role in receptor interaction, since the loss in biological activity is similar upon replacement of proline with either alanine or D-proline, without a large alteration in conformation. In position 3, the ring clearly does not have a function in receptor interaction, since the alanine analog is fully potent. The case of the 2 position is less clear, since replacement by D-proline (which causes a large conformational change) or by alanine (which lacks the ring) causes a similar diminution of biological activity.

One might hope to shed additional light upon this question by examination of the biological activities and CD spectra of BK analogs containing substituted prolines. For this purpose 4-hydroxyproline is a logical candidate, since the size, hydrophilic character, and hydrogen bonding ability of the hydroxyl group might be expected to influence the biological activity and conformation of the peptides. These hydroxyproline analogs of BK are also of synthetic interest. Although hydroxyamino acids can often be used in classical peptide synthesis (where equimolar

† A preliminary report of this work has appeared; see ref 1.