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ara-Cytidine Acylates. Use of Drug Design Predictors in Structure–Activity Relationship Correlation[1,2]

W. J. Wechter,* M. A. Johnson, C. M. Hall, D. T. Warner, A. E. Berger,[3] A. H. Wenzel,[4] D. T. Gish, and G. L. Neil

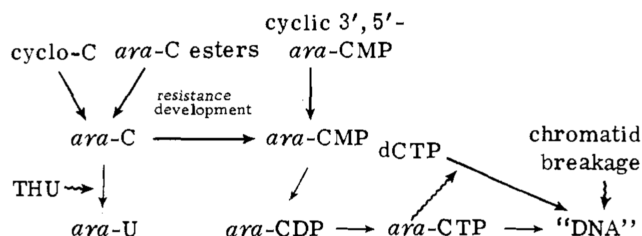
Research Division, The Upjohn Company, Kalamazoo, Michigan 49001. *Received September 4, 1974*

This manuscript is one of a series of investigations into modifying the pharmacologic properties of the antitumor, antiviral, and immunosuppressive nucleoside *ara*-cytidine (cytarabine, Cytosar). The present paper summarizes our studies on depot ester derivatives of the nucleoside. We are able to predict with reasonable accuracy the biological activity as measured by increased life span in the L1210 leukemic mouse from a combination of two predictor variables: (1) the solubility of the ester in water and (2) its rate of hydrolysis by the mixed esterase system of animal plasma. We have tried unsuccessfully to correlate enzymatic hydrolysis rates with an alkaline hydrolysis model. Calculated Hansch partition (p) values had a correlation of $r = 0.86$ with water solubility. These p values had no additional predictive value. Based on our results, two esters were selected for clinical trial in cancer and rheumatoid arthritis.

Since the demonstration of the antitumor, antiviral, and immunosuppressive effects of the nucleoside *ara*-cytidine (Cytosar, cytarabine) (for leading references, see ref 1), we have attempted to modify the pharmacological properties of this unique nucleoside. We have tried to increase its potency, modify its catabolism to *ara*-uridine, develop depot and orally active forms, increase its specificity, obviate resistance development, modify its biological profile, and understand its mechanism of action. Some success has been achieved in reaching all of these objectives.[2] This paper deals exclusively with our development of a depot form of the drug employing *in vitro* correlates for the design of a drug for clinical application in cancer and rheumatoid arthritis, the latter to be effective as a locally administered (intraarticular) immunosuppressive agent in rheumatoid joints.

Early efforts by members of our group with synthetic dinucleoside phosphates containing *ara*-C led to minor increases in potency[5,6] and no apparent improvement in specificity. Synthesis of the potent cytidine aminohydrolase inhibitor tetrahydrouridine[7] provided a compound which, given in combination with *ara*-cytidine, afforded increased plasma half-life of the drug by blockage of the facile catabolic pathway *via* deamination to *ara*-uridine. The major impetus to the present work was the dramatically enhanced activity of the 5′-adamantoate ester[8] over *ara*-cytidine itself when administered as a single dose. This, in turn, led to the examination of a host of acylates at C-2′, C-3′, and C-5′. In three earlier publications[9-11] the synthesis and biological activity of the majority of the esters were reported. In this paper we attempt to analyze the structural requirements for activity for the purpose of the design and synthesis of additional esters employing our predictor correlates. We also prepared some diesters for evaluation. From an understanding of the biochemistry of *ara*-cytidine (Scheme I) and our rudimentary theses for drug design, we

Scheme I



felt that the four most important variables with which we would have to deal were (1) the deamination to the inactive *ara*-uridine esters; (2) the dissolution of the drug *in vivo*; (3) its transport and distribution; and (4) its *in vivo* hydrolysis to the active species, *ara*-cytidine, which necessarily must be the precursor of the active drug (see Scheme I) *ara*-CTP in all cases. Early work with the deaminase enzyme of human serum established that the esters were not substrates. Consequently, this factor could be discarded as a design parameter.[9] As an *in vitro* correlate of dissolution we chose, as an approximation, water solubility. The Hansch p values were selected as the corresponding correlate of transport and distribution. Our most thoroughly in-

Table I

Compd	No.	% ILS,[h] single dose of ~200 mg/kg	Solubility in H_2O, μg/ml[j]	Hydrolysis: Mouse plasma, 10^3K (M^{-1})	Hydrolysis: Human plasma, $10K$ (M^{-1})	Hydrolysis: Human synovial fluid, $10K$ (M^{-1})	Alkaline, K (M^{-1})	Log p (pH 7): Calcd	Log p (pH 7): Exptl	Ref.
ara-Cytidine	**1**	<25	Very soluble						−2.1	
5′-Palmitate	**2**	>300	1.5–2	(>139)	(2)		0.2	4.8	2.1	*a*
5′-Laurate	**3**		20–27	(300)			(0.3)	3.8		*e*
5′-Adamantoate	**4**		27	139	2.0	1.5	0.02	2.8		*b*
5′-Stearate	**5**	200–300	1	(>10.5)	(>0.05)	(0.3)	0.27	5.8		*c*
3′-Stearate	**6**		1	13	0.33		0.22	5.8		*c*
5′-PSBPP[g]	**7**		23	1.8	0.35	(0.18)	0.31	6.1		*e*
5′-Benzoate	**8**		75–90	321	31	6.6	0.18	0.4	0.5	*a*
5-*p*-Methoxybenzoate	**9**		165	35	1.2			0.4		*a*
3′,5′-Dipalmitate	**10**	40–80	<1		0			>8		*c*
2′-Stearate	**11**		(~1)[i]	0.78	<0.1			5.8		*c*
5′-(2,6-Dimethylbenzoate)	**12**		77	(<0.1)	(<0.1)	(<0.1)	(<0.001)	1.4		*a*
3′-Benzoate	**13**		(~75)	11.2	24	4.8		0.4		*c*
5′-Triethyl acetate	**14**		160	0.3	(0.01)	(0.01)	(<0.001)	1.4	1.2	*e*
5′-Pivalate	**15**		1600–1900	2.1	0.21	(<0.01)	0.06	0.3	0.1	*a*
O^2-2′-Cyclocytidine	**16**		Very soluble						−2.3	*d*
5′-Diethyl acetate	**17**		(>200)	0.3	0.2		0.006	0.6		*e*
2′,3′-Dibenzoate	**18**	<25	Insoluble	(0.03)	0.1	(0.06)		>8		*c*
3′,5′-Dibenzoate	**19**		Insoluble	(0.05)	(<0.1)	(<0.01)		>8		*e*
5′-(2,4,6-Trimethylbenzoate)	**20**		14–38	(<0.1)	(<0.1)	(<0.1)	(<0.001)	1.9		*a*
2′-Benzoate	**21**		(~75)	(0.03)	0.12	(<0.01)		0.4		*c*
5′-Octanoate	**22**		170–220	321	321	95	0.28	1.8	1.6	*a*
5′-Cyclohexylcarboxylate	**23**		560	116	139	82	0.15	0.6	0.8	*a*
5′-(3,4,5-Trimethoxybenzoate)	**24**		1500	(0.67)	(0.05)	(0.17)	0.16	0.5		*a*
5′-β-Chloropivalate	**25**		2700	1.5	(0.03)	(0.08)	0.21	0.2		*a*
5′-(2,4,6-Triisopropylbenzenesulfonate)	**26**		1	(<0.1)	(0.04)	(0.06)	1.7	(5.8)		*a*
5′-Cyclobutanecarboxylate	**27**		8000	87	76	6		−0.1		*a*
5′-Isobuteryl	**28**		20,000	9.9	7.6	0.8	0.34	0.3	0.3	*a*
5′-Acetate	**29**		Very soluble	1.7	10.8	0.07	1.1	−1.2	−1.4	*a*
5′-(*p*-Toluenesulfonate)	**30**		(soluble)				Very fast			*a*
5′-(3-Quinuclidinoyl)	**31**		(soluble)	(<0.1)	0.94	(<0.01)			−1.0	*a*
5′-Isovalerate	**32**		(soluble)				0.093	0.1		*e*
5′-Nicotinate	**33**	25–40	(soluble)	5.1	9.9	1	2.8	−0.64	−0.6	*a*
5′-(2,3,3-Trimethylbuteryl)	**34**	[f]	(~200)				<0.001	1.4		*e*

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vestigated parameter was the hydrolysis correlates which included crude enzyme hydrolysis by mouse and human plasma, human synovial fluid, and finally alkaline hydrolysis as a model of enzymatic hydrolysis. The results of these studies are summarized in Table I.

From earlier work[8-10] it was concluded that low water solubility was a necessary, but not sufficient, requirement for a high order of biological activity and that enzymatic hydrolysis was required. We have now refined the predictor variables so that we can predict potency with a high degree of success from a combination of solubility and enzyme hydrolysis rates. Based on this information two compounds were selected for study in man, the purpose being to furnish a depot form of *ara*-cytidine which would provide, on a continuous basis, a minimum therapeutic level of drug, either systemically (in cancer) or locally (in rheumatoid joints).

The predictor variables that we evaluated included the partition coefficient, water solubility, and four hydrolysis rates. Some of the values for these predictor variables were estimated rather than experimentally determined. With the exception of the partition coefficient, the estimated value for a predictor variable was based on the experimental determination of another compound, structurally the

same except for a modification that was not expected to influence the experimental determination (*i.e.*, 2,4,6-trimethyl- *vs.* 2,6-dimethylbenzoate). Statistical analysis does not differentiate between these estimated and experimental values of the predictor variables.

The partition coefficient was experimentally determined for 11 compounds and calculated for 25 compounds. The relationship between the experimental and calculated values is very good, $r = 0.99$ excluding the palmitate ester **2** and $r = 0.9$ including this ester for those compounds for which both values were obtained. The Hansch[12] calculation of the log partition coefficient is not reliable for compounds with high partition coefficients. Owing to its limited solubility, the partition coefficient of the palmitate ester **2** was measured as 5′-*O*-palmitoyl-*ara*-cytidine-2-^{14}C and proved to be 2.1 rather than the calculated 4.8. This experimental p value should have been associated with a C_9 acid ester. Consequently, we suggest that this difference may be due to folding or balling of the fatty acid side chain as well as micelle formation in the highly lipophilic derivatives. Consequently, compounds **2–6** and **11** are not subject to calculated p values. The correlation of log p with biological activity was limited to the palmitate plus the 19 esters whose log p value was less than 2.0. For these 20 compounds no significant relationship with activity (% ILS) emerged, nor could the most interesting group of compounds **3–6** and **11** be included. The correlations of all predictor variables were determined using the logarithms of their values. Values known (by experiment) to be less than or greater than a particular value were excluded from estimation of the correlation coefficients (r) because of the difficulty with dealing with them statistically. On the other hand, using the calculated p values there was a high degree of correlation between water solubility and log p. The correlation coefficient was $r = 0.86$ based on 18 pairs and was significantly different from 0 at a significant level less than 0.01. Consequently, during the course of the selection of straight chain esters for synthesis, the laurate ester **3** was prepared as a solubility analog of the adamantoate ester **4**. Since the log solubility of the adamantoate ester fell midway between the C-8 octanoate **22** and C-16 palmitate **2** esters, the C-12 laurate ester **3** which should have a calculated intermediate log p was prepared. Its solubility was close to that predicted. The approximations of this procedure are not consistent since the log p of the laurate **3** and estimated log p of the adamantoate **4** ester differ by one log unit.

Table II. Correlations among Hydrolysis Predictor Variables

	Alkaline	Mouse plasma	Human plasma	Human synovial
Alkaline	1			
Mouse plasma	−0.08 (16)	1		
Human plasma	0.11 (16)	0.76 (19)[a]	1	
Human synovial	−0.35 (13)	0.86 (15)[a]	0.89 (15)[a]	1

[a]Significantly different from 0 at 0.01.

Table III. Formation of Response Groups

Assigned score	Log p	Solubility, μg/ml	% ILS
1	>3.0	<5	<25
2	2.1–3.0	5–30	25–100
3	1.1–2.0	30–100	100–300
4	0.1–1.0	100–300	>300
5	<0	>300	

Table IV. Relationship of % ILS with Solubility[a]

% ILS score		1	2	3	4	5
	4	1	2	0	0	0
	3	2	1	1	1	0
	2	2	0	2	1	3
	1	3	1	1	2	10

(Columns 1–5: solubility score)

[a]The number of analogs with a particular % ILS score and solubility score is indicated.

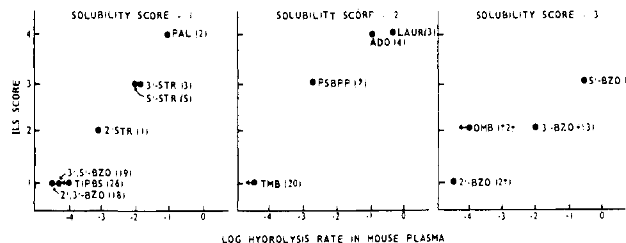

Figure 1. Log hydrolysis rate in mouse plasma (M^{-1}) *vs.* increase in life span (ILS) score for the L1210 infected mouse.

Because the initial biological evaluation of these compounds was in the mouse, we chose to evaluate the ester hydrolysis by mouse plasma as a predictor variable. Since the compounds were to be used in man, we also evaluated hydrolysis rates by human plasma (cancer studies) and human synovial fluid from an arthritic patient (rheumatoid arthritis studies). In an attempt to model these mixed enzyme studies with a chemical equivalent we determined first-order alkaline hydrolysis rates of the esters at high pH (see Table I).

Evaluation of these data revealed that alkaline hydrolysis rates afforded no correlation with enzymatic hydrolysis. Esterase hydrolysis by human plasma, mouse plasma, and human synovial fluid had a high degree of correlation with one another and thus the mouse plasma hydrolysis serves as an exemplary case (see Table II).

Earlier work[8,9,13] established that low water solubility was a necessary requirement for a high order of biological activity (see Tables II–IV). If one admits only compounds with the same low solubility score, one finds that the mouse plasma hydrolysis rate is an important predictor of biological activity (see Figure 1). Because the log partition coefficient is closely correlated with the log solubility values ($r = 0.86$), the partition coefficient serves just as well in the preceding argument. We use solubility because it has a closer logical connection with the dissolution of the drug. It should be noted that the diesters and the 3′- and 2′-esters generally were not comparable to the 5′-esters in biological activity. It now seems apparent that their lower activity is probably due to attenuation of their hydrolysis rates, generalizing from the limited numbers of such esters. This is most evident with the esters in solubility classes 1 and 3 in Figure 1.

Since no animal preparation is a good predictor of the clinical efficacy of *ara*-C owing to the complex biochemical parameters (Scheme I) which differ greatly between species[8–10] we chose, on the basis of these data, two compounds for further evaluation in man. In order to get the widest divergence of chemical species, we chose the most highly active compounds from low solubility high activity groups 1 and 3 (Figure 1), namely, the palmitate **2** and ben-

Table V

ara-C 5′-ester	Composition	Reagent	Recrysn solvent	Mp, °C	Yield, %	Analyses
Laurate (3)	$C_{21}H_{35}N_3O_6 \cdot 0.5H_2O$	AC[a]	MeOH	144–148	56	C, H; N[b]
Stearate (5)	$C_{27}H_{47}N_3O_6$	AC	MeOH	141–143.5	57	C, H, N
dl-2-(*p*-Isobutyl-phenyl)propionyl (7)	$C_{22}H_{29}N_3O_6 \cdot 0.5H_2O$	AC	CH_3CN–H_2O	170–173	43	C, H, N[c]
2,3,3-Trimethyl-butyrate (34)	$C_{16}H_{25}N_3O_6$	AC	EtOH	228–230	94	C, H, N
Diethyl acetate (17)[e]	$C_{15}H_{23}N_3O_6$	AC	*n*-BuOH–H_2O	240	54	C, H, N[d]
Isovalerate (32)[e]	$C_{14}H_{21}N_3O_6$	AC	EtOH	214–215	45	C, H, N
Triethyl acetate (14)	$C_{17}H_{27}N_3O_6$	AC	EtOH	261 dec	71	C, H, N

[a]Acid chloride. [b]N: calcd, 9.67; found, 9.00. [c]H_2O (KF): calcd, 2.05; found, 2.01. [d]Dried at 120° *in vacuo* to remove H_2O. [e]Hexamethylphosphoric triamide was used as reaction solvent.

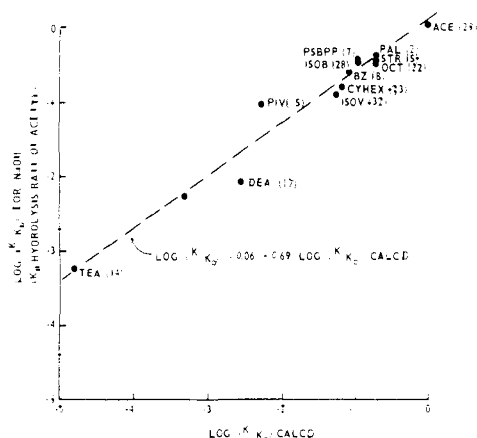

Figure 2. Log [K (ester)/K (acetate)] for esters of ethanol *vs.* log [K (ester)/K (acetate)] observed.

zoate 8 esters. Their high hydrolysis rates ensured that as the ester dissolved in the biophase they would be hydrolyzed at a sufficient rate to prevent excretion of the unchanged ester from the plasma (systemic administration) or arthritic synovium (local administration) before phosphorylation to the active nucleotide *ara*-CTP (see Scheme I). Hopefully, these two compounds will serve as depot forms of *ara*-cytidine which will provide the desired minimum therapeutic blood or synovial level of the *ara*-cytidine over an extended period of time. To this end biochemical pharmacology studies of 2-^{14}C derivatives of the two esters have begun in man.

Alkaline Hydrolysis. The hydrolyses were carried out in an aqueous acetone solution and a sufficient excess of alkali was used to obtain pseudo-first-order reaction rates. Results are tabulated in Table I. Generally, the observed rates were unexceptional. It was interesting that the alcohol *ara*-cytidine did not behave as a simple alcohol. When log K/K_0 (obsd) was plotted against the (K/K_0) theory values,[14] a straight line was generated (Figure 2) which did not have a slope of 1, as would be expected if the nucleoside were essentially equivalent to ethanol. Rather, all rates were faster than anticipated yielding a least-squares line with a slope of 0.7 and a standard deviation of 0.05. This observation suggested participation of the nucleoside in the hydrolysis reaction. The most likely explanation would be expected to be the participation of the 3′-OH of the sugar in internal solvation of the transition state for attack of the OH^- at the ester carbonyl similar to that observed by Bruice for rate acceleration by neighboring hydroxyls.[15]

Methods. Calculations of Log *p* Values. Log *p* values were calculated by the method of Hansch[12] using the known experimental log *p* of *ara*-C (unpublished data, W. Morozowich, Upjohn Co.) and values from the literature.[12] 0.2 was subtracted for each branch in a chain, and 0.5 was added for each methyl or methylene group, except when the structure was closed, when a value of 0.4 was added.

Experimental Determination of Log *p* Values. Saturated solutions of octanol and water (O/W), and water and octanol (W/O), were obtained by shaking together equal volumes of 1-octanol (Burdick and Jackson Laboratories, Muskegon, Mich.) and distilled water for 24 hr. The phases were allowed to equilibrate at least 1 hr before separation and, once separated, were allowed to stand overnight before use. Samples of esters were dissolved in the solvent (O/W or W/O) in which they were most soluble. An aliquot of this ester solution was taken, and an equal amount of the other solvent was added to it. The combination was shaken for 15 min (30 min in the case of the adamantoate and palmitate esters). Samples were taken from each layer and, after equilibration, the uv spectra (325–260 mμ) for both layers were determined against an appropriate blank on a Cary 15 spectrophotometer. The maximum absorption was read and the partition coefficient determined: from Beer's law ($A = bc$, where A is the absorbance, ϵ the molar extinction coefficient, b the cell length in cm, and c the concentration in moles per liter) and the definition of the partition coefficient

$$P = c_{O/W}/c_{W/O} = \frac{(A_0/E_w b_w)}{(A_w/E_0 b_0)}$$

or the cell length constant at 1 cm

$$P = (A_0/E_w)/(A_w/E_0)$$

Molar extinction coefficients, or molar absorptivities, ϵ, used in calculating the partition coefficients, were either determined experimentally by measuring the absorbance of known concentrations of ester in the appropriate solvent or, in the case of the adamantoate and palmitate esters, were estimated. $E_{W/O}$ for ten esters was on the average 400 higher than the $E_{O/W}$ for the same esters. $E_{O/W}$ for the adamantoate and palmitate had been experimentally determined, and the $E_{W/O}$ for each was assumed to be 400 higher than the appropriate $E_{O/W}$.

Enzymatic Hydrolysis. Human plasma or rheumatoid synovial fluid (obtained from Dr. Roland R. Springgate, Borgess Hospital, Kalamazoo, Mich.) samples were preincubated (37°; 30 min) with 20 μg of tetrahydrouridine/ml of sample [THU, NSC 112907, 1-(β-D-ribofuranosyl)-4-hydroxy-3,4,5,6-tetrahydropyrimidin-2-(1H)-one]. THU was employed to inhibit deamination of the hydrolysis product *ara*-C to *ara*-U.[16]

Preliminary experiments had shown the esterase activity of full strength mouse plasma to be too high to evaluate hydrolysis over feasible time intervals. For this reason, mouse plasma was diluted 1:10 with 0.1 *M* NH_4Cl buffer at the pH of the plasma (7.45). Dilute mouse plasma samples were also preincubated 30 min at 37° with 20 μg/ml of THU.

At zero time, 2 mg/ml solutions of esters in dimethyl sulfoxide (DMSO, Burdick and Jackson) were added to yield an initial ester concentration of 20 μg/ml (and a concentration of DMSO of 1%). At designated time intervals, aliquots were removed and added to 2 vol of absolute ethanol to precipitate proteins. Protein precipitate was removed by centrifugation, and the supernatants were assayed using the *Streptococcus faecalis* assay for *ara*-C.[16]

Standard *ara*-C samples were prepared in supernatants obtained by adding a double volume of absolute ethanol to a volume of the appropriate plasma or synovial fluid. *ara*-C was added to the supernatant in concentrations ranging from 0.0625 to 8 μg/ml. A control sample containing no drug was also assayed. After appropriate incubation, diameters of zones on inhibition were measured. Linear regression analysis of log *ara*-C concentrations *vs.* zone diameter provided the standard curve for determination of *ara*-C concentration in the samples. The lower limit of sensitivity of this assay was 0.0625 μg/ml. Neither 1% DMSO nor 20 μg/ml carboxylic acids (β-chloropivalic, cyclobutanecarboxylic, cyclohexanecarboxylic, 3,4,5-dimethoxybenzoic, or pivalic) was inhibitory under assay conditions (assuring that growth inhibition was due to the *ara*-C itself). Appropriate controls were also run in NH_4Cl buffer (pH 7.45) to determine rates of nonenzymatic hydrolysis. In all cases, rates were corrected for this factor.

Initial rates for the reactions were determined from plots of *ara*-C concentration *vs.* time. Semilogarithmic plots of ester concentration *vs.* time (at any time point) showed hydrolysis to follow first-order kinetics.

In most cases, half-lives were determined directly from the semilogarithmic plots of ester disappearance, and first-order rate constants were calculated.

In cases where the half-life was greater than 500 min, it was more convenient to calculate K and $t_{1/2}$ from the initial rate.

Antitumor (L1210) Activity. Female BDF_1 mice (20 g) were inoculated intraperitoneally (ip) on day 0 with 10^6 L1210 cells per mouse in a volume on 0.1-ml sterile saline. On day 1 following inoculation, compounds were administered ip as solutions in sterile saline or suspensions[17] in sterile Vehicle 122 (0.25% aqueous methylcellulose, The Upjohn Co.). Deaths were recorded daily and mean days of death calculated for the test groups of six or eight animals. Control animals (30 per group) were inoculated and treated with vehicle only.

$$\% \text{ ILS} = \% \text{ increase in life span} = \left(\frac{\text{test group mean day of death}}{\text{control group mean day of death}} \times 100\right) - 100$$

An increase in life span of 25% is considered significant for this system, and any compound producing a per cent ILS of greater than or equal to 25 is considered active.

Determination of Water Solubilities. The derivatives were added to water at room temperature and the suspensions stirred for greater than 1 hr. The optical densities of the saturated solutions obtained after Millipore filtration (0.45 μ) were measured at their absorption maxima (*ca.* 270 mμ). The molar extinction coefficients in water ranged from 7.9×10^3 for the adamantoate to 9.7×10^3 for the benzoate, with the rest falling between 9.2 and 9.5×10^3 (cytarabine was 9.45×10^3). A general molar extinction coefficient of 1×10^4 was then used to calculate the concentration of each derivative and their solubilities were expressed in micrograms per milliliter. Any compound soluble in excess of 2000 μg/ml was considered soluble. For the "insoluble" esters (<200 μg/ml), the ester was first stirred for several hours with water in order to remove any free *ara*-C contaminant which would greatly influence the apparent solubility.

Experimental Section

5'-Esters of *ara*-C. The 5'-esters of *ara*-C were prepared by the general procedure of Gish, *et al.*,[9] using *ara*-C · HCl in DMF or DMA and the appropriate acid chloride or acid anhydride. The compounds are listed in Table I. In two instances (see Table V) hexamethylphosphoric triamide was used as a solvent instead of DMF or DMA.

3',5'-Dipalmitoyl-*ara*-cytidine (10). The hydrochloride of *ara*-C (200 g, 0.715 mol) was dissolved in 1.5 l. of dimethylformamide at room temperature. Palmitoyl chloride (393 g, 1.43 mol) was added with stirring. The reaction temperature warmed to 38° and a clear solution resulted. On cooling, precipitate formed and overnight the reaction mixture formed a thick slurry. Dilution with 6 l. of 1:1 ethyl acetate–ether filtered the solid which was washed with ethyl acetate–ether and then with ether. The crude solid was stirred with 4 l. of 0.5 *N* $NaHCO_3$, filtered, washed with water, and dried *in vacuo*. Recrystallization from 5 l. of MeOH gave 180 g. An additional 68 g was recovered from the filtrate: total yield 248 g (51%).

The main product (R_f 0.50, $CHCl_3$–MeOH 9:1) had a minor impurity (R_f 0.87) and other trace components. These were removed by chromatography (silica gel) eluting with $CHCl_3$ to remove the impurity and then with $CHCl_3$–EtOH to remove the product. After an additional recrystallization from MeOH, the 3',5'-dipalmitoyl-*ara*-cytidene melted at 111–112° after shrinking to an opaque glass at 98°: nmr H-1' band at δ 6.1, H-5 doublet at 5.8, H-3' complex peak at 5.1, H-5' α and β, complex peak at 4.25. *Anal.* ($C_{41}H_{73}O_7N_3$) C, H, N.

3'-*O*,5'-*O*-Dibenzoyl-*ara*-cytidine (19). An attempt to make this compound directly from *ara*-C · HCl with excess benzoyl chloride, as in the case of the dipalmitoyl derivative above, was unsuccessful. A successful synthesis started with 2,2'-anhydro-*ara*-C · HCl.[14]

(a) 3'-*O*,5'-*O*,N^4-Tribenzoyl-*ara*-cytidine Hydrochloride (35). 2,2'-Anhydro-*ara*-C · HCl (5.22 g, 0.02 mol) suspended in 160 ml of anhydrous pyridine was allowed to react with 9.0 g (0.063 mol) of benzoyl chloride added with stirring during 2 hr. After 24 hr at 25° a clear solution resulted, but tlc indicated three components (R_f 0.28, 0.53, and 0.82, $CHCl_3$–MeOH 85:15). Upon heating at about 85° (2 hr), most of the material was the fast-moving component (R_f 0.82) which was concentrated *in vacuo* to remove pyridine and repeatedly concentrated several times with addition of EtOH. The semisolid residue was slurried with 50 ml of MeOH, filtered, washed, and recrystallized from MeOH: wt 4.33 g; mp 197.5–198.5°. An additional 3.05 g of material was recovered from the filtrate: yield 7.38 g (64%); nmr H-5 and H-6 doublets at δ 7.38 and 8.3, H-1' doublet at 6.3, H-2' and H-3' complex bands at 5.82 and 5.35, H-5' α and β, and H-4' band at 4.8, about 19 aromatic protons (2 vinyl). *Anal.* ($C_{30}H_{25}O_8N_3 \cdot H_2O$) C, N; H: calcd, 4.75; found, 4.25.

(b) 3'-*O*,5-*O*-Dibenzoyl-*ara*-cytidine from 35. The 3'-*O*,5'-*O*,N^4-tribenzoyl-*ara*-cytidine was hydrolyzed selectively at the N^4-benzoyl group using the procedure of Letsinger, *et al.*[13] 3'-*O*,5'-*O*,N^4-Tribenzoyl-*ara*-cytidine (1.5 g, 0.0026 mol) was dissolved in 15 ml of pyridine–acetate acid (4:1). About 0.35 ml of hydrazine hydrate was added. The reaction mixture was stirred 1 hr at 25° and refrigerated overnight; then it was concentrated *in vacuo* to remove pyridine and acetic acid. The residue was dissolved in CH_2Cl_2, washed with water, dried with anhydrous Na_2SO_4, and purified by chromatography on silica gel, eluting with CH_2Cl_2–MeOH. About 700 mg of product was recovered from the column which was recrystallized from MeOH–H_2O: wt 538 mg (44%); mp 247–248° dec. A yellow-colored impurity was removed by an additional chromatography on silica gel. The colorless product was recrystallized from MeOH–ethyl acetate or MeOH–H_2O: mp 247–248° dec; nmr H-1' doublet centered at 6.3, 4-NH_2 proton buried in aromatic peak but evident at *ca.* 7.5 by D_2O exchange, H-5 doublet centered at 5.85, H-3', complex peak at 5.25, and H-5'

α and β, 4.72, about 13 aromatic protons. *Anal.* ($C_{23}H_{21}O_7N_3 \cdot H_2O$) H, N; C: calcd, 58.84; found, 59.28.

Hydrolysis Procedure. Exactly 0.4 mmol of the ester was dissolved in 200 ml of acetone contained in a 250-ml volumetric flask. The solution was diluted to the mark with H_2O and thoroughly mixed to give a 0.0016 *M* solution of the ester. Aliquots (25 ml) were transferred *via* a volumetric pipet to 50-ml erlenmeyer flasks, the flasks were stoppered, and the contents were equilibrated in a bath maintained at 40 ± 0.2°. Into each aliquot was rapidly pipetted exactly 5 ml of 0.100 *N* NaOH. This gave an initial concentration of the ester of 0.00133 *M*. The mixture was stirred magnetically, and at time intervals the hydrolysis was stopped by rapidly pipetting exactly 5 ml of 0.100 *N* HCl into the flask. The contents were cooled to room temperature, a few drops of phenolphthalein solution were added, and the mixture was titrated to the end point with 0.100 *N* NaOH. The amount required to reach the end point, minus the blank value, was equivalent to that consumed during the hydrolysis. Blank values were determined by pipetting 5 ml of the standard 0.1 *N* NaOH into 25-ml aliquots of the acetone–H_2O solvent, pipetting into this mixture 5 ml of the standard 0.1 *N* HCl, and titrating this mixture to the phenolphthalein end point with the standard 0.1 *N* NaOH. The same pipets were used for the blanks that were used for the hydrolysis.

Molar concentrations of the ester at time intervals during the hydrolysis were calculated from the hydrolysis data, and the $-\log C$ values were plotted as a function of time as shown in Scheme I. The hydrolysis constant k was calculated from the relationship $k = 2.3 \log C/t$.

Determination of Partition for the Palmitate Ester 2. ^{14}C-Palm-*O*-*ara*-C (2) (19.2 μCi/mg, labeled in the 2 position of the pyrmidine ring) was added to a stoppered erlenmeyer flask containing 15 ml each of 1-octanol and sodium phosphate aqueous buffer (pH 7.0, 0.035 *M*, ionic strength 0.1). After vigorous shaking (37°, 1 hr) and subsequent phase separation, radioactivity in 1.0-ml aliquots of each phase was determined using a liquid scintillation spectrometer. The aqueous phase was removed and replaced with fresh buffer. The shaking procedure was repeated with fresh buffer until a constant partition coefficient was obtained. The value obtained was 127.3 (octanol–water) and the octanol concentration of Palm-*O*-*ara*-C (2) in octanol at equilibrium was 1.5 μg/ml.

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Linear Regression Analysis of Inhibitory Potency of Organic Disulfides against *Histoplasma capsulatum*[†]

L. J. Schaad,* R. H. Werner,[‡] Lynn Dillon, Lamar Field,* and C. Emory Tate

Department of Chemistry, Vanderbilt University, Nashville, Tennessee 37235. *Received July 25, 1974*

The Free–Wilson equations are derived for the case of symmetrical substitution and are applied, in four modifications, to *in vitro* inhibitory activity of 77 organic disulfides against *Histoplasma capsulatum*. Substituent constants are listed to aid in the design of new inhibitory agents against this human pathogen (and perhaps other fungal organisms).

As part of a search for improved inhibitory agents against *Histoplasma capsulatum*, the causative organism of histoplasmosis, a regression analysis of the *in vitro* activity against *H. capsulatum* was carried out for 77 organic disulfides. There are two main approaches to the problem of correlating biological activity with chemical structure. The one, due to Hansch,[1] correlates biological activity with other physical parameters, especially the partition ratio between octanol and water. The other, by Free and Wilson,[2] estimates biological activity from empirically fitted substituent constants. Craig[3] gives a readable comparison of the two. A recent chapter by Cammarata and Rogers[4] reviews applications of these methods and contains a useful discussion of the physical basis for the mathematical models.

Since the compounds to be considered here are all disulfides with varying substituents, and since we have no knowledge of the details of the drug action against *H. capsulatum* and cannot reasonably postulate a correlation with any particular physical parameter, the Free–Wilson approach seemed the more applicable.

Mathematical Background. Free and Wilson defined their activity parameters relative to the average activity of the set of compounds studied. This is only one of several equivalent methods. To see how these arise from a linear

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