Characteristics of the new 1-phenyl-2-arylhydrazono-1,2,3-butanetriones are summarized in Table II.

2-Amino-4-methyl-6-phenylpyrimidine.—Guanidine nitrate (2.5 g, 0.02 mol) was added to 1-phenyl-1,3-butanedione (3.2 g, 0.02 mol) containing 10 *N* NaOH (10.0 ml) and MeOH (20.0 ml). The mixture was stirred for 10 hr at 50–60° and left for another 12 hr at room temperature. The precipitated 2-amino-4-methyl-6-phenylpyrimidine was collected and washed with MeOH and hot H_2O. It was recrystallized from MeOH (2.3 g, 65%), mp 171°. *Anal.* ($C_{11}H_{11}N_3$)C, H, N.

2-Amino-4-methyl-6-phenyl-5-(2,4-dimethoxyphenylazo)-pyrimidine. Method A.—The diazotized 2,4-dimethoxyaniline, free from HNO_2 was added to a well-cooled and stirred solution of 2-amino-4-methyl-6-phenylpyrimidine (3.7 g, 0.02 mol) in AcOH (45.0 ml) containing sufficient NaOAc so as to maintain the reaction mixture at pH 6–7. The mixture was stirred for another 6 hr at 0–5° and left for 24 hr at room temperature. The product thus precipitated was collected, washed well with H_2O, and recrystallized from DMF–EtOH (3.6 g, 66%) as golden yellow plates, mp 160°. *Anal.* ($C_{19}H_{19}N_5O_2$) C, H, N.

Method B.—Guanidine nitrate (2.5 g, 0.02 mol) was added to 1-phenyl-2-(2,4-dimethoxyphenyl)hydrazono-1,2,3-butanetrione (6.5 g, 0.02 mol) which in turn was prepared by coupling diazotized 2,4-dimethoxyaniline with 1-phenyl-1,3-butanedione, containing 10 *N* NaOH (10.0 ml) and MeOH (20.0 ml). The mixture was stirred for 12 hr at 60–70° and left for another 12 hr at room temperature. 2-Amino-4-methyl-6-phenyl-5-(2,4-dimethoxyphenylazo)pyrimidine precipitated, was collected, and was washed successively with MeOH and hot H_2O. It was recrystallized from DMF-EtOH (2.7 g., 51%) as golden yellow plates, mp 160°.

By similar procedures, several 2-amino-4-methyl-6-phenyl-5-arylazopyrimidines were prepared; they are summarized in Table I.

Acknowledgment.—The authors are greatly indebted to Drs. H. B. Wood and H. W. Bond of the Cancer Chemotherapy National Service Center for their cooperation and for making the screening data available. We are also thankful to Professor W. U. Malik, Head of the Chemistry Department, for providing the necessary facilities for this work and the C.S.I.R., New Delhi (India) for a Junior Research Fellowship (held by R.A.S.).

Synthesis of 5-Iodo-3-indolylphosphodiesters of 5-Fluorodeoxyuridine As Possible Chromogenic Cancer Chemotherapeutic Agents[1]

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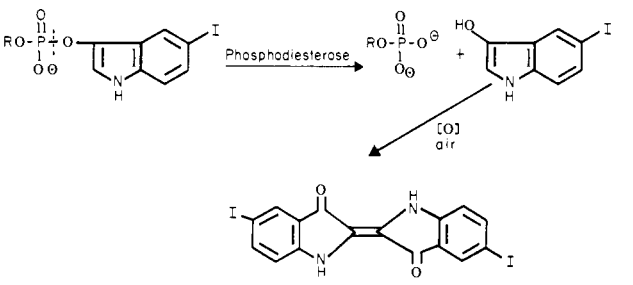
In the design and synthesis of anticancer agents based on enzyme rationale,[2] it would be desirable to have one of the enzymatically hydrolyzed products as a chromogen so that the fate of such enzyme-mediated drugs can be followed. Previously, we reported the synthesis of several tetrazolium mustards which could be reduced *in vivo* to a more toxic, colored formazan mustard.[3] The present paper reports the incorporation of such a concept to a known useful antimetabolite-type anticancer agent, utilizing the phosphodiesterase principle reported previously,[4] and as illustrated in Scheme I.

SCHEME I

Principle of Phosphodiesterase



5,5'-diiodoindigo (purple-blue dye)

Through detailed study by Heidelberger and his associates, 5-fluorodeoxyuridine 5′-phosphate (FUDRP) was shown to be the active antitumor metabolite when either 5-fluorouracil (FU) or 5-fluorodeoxyuridine (FUDR) were used in the management of several types of cancer.[5] However, the use of the nucleotide, FUDRP, itself has not been an easy task in cancer research because of the facile hydrolysis by either 5′-nucleotidase or an acid phosphatase. With the success we have had in the synthesis of 5′-iodoindolyl phosphodiesters of deoxythymidine,[6] it was considered of interest to synthesize the 5′- and the 3′-(5-iodoindolyl)phosphodiesters of 5-fluorodeoxyuridine, **1** and **2**, respectively, as possible chromogenic anticancer agents. Upon enzymatic hydrolysis, they could liberate the 5′- and 3′-nucleotides, and thus provide a known active anticancer agent, especially in the case of the 5′ derivative.

The synthesis of the 5′ and the 3′ derivative is illustrated in Scheme II.

The starting materials, 5′-*O*-trityl-5-fluorodeoxyuridine and 3′-*O*-acetyl-5-fluorodeoxyuridine, were prepared by a procedure similar to that of Remy, Sunthanker, and Heidelberger,[7] based on methods of Michelson and Todd,[8] and Gilman and Khorana[9] for deoxythymidine derivatives. 5-Iodoindolyl-*N*-acetate was prepared according to the method of Rabiger, *et al.*[10] The phosphodichloridate was prepared in dry pyridine and used directly for the reaction. The product was purified and isolated as the ammonium salt on a Sephadex column with a linear gradient of 0.02 to 0.3 *M* NH_4HCO_3. The purity of the product was checked by elemental analysis as well as R_f values with paper chromatography in three different solvent systems. The ir of the 3′ derivative differs from the 5′ only at

(1) This work was supported by AEC Research Grant AT(30-1)3784 and U. S. Public Health Service Research Grant CA-07339.

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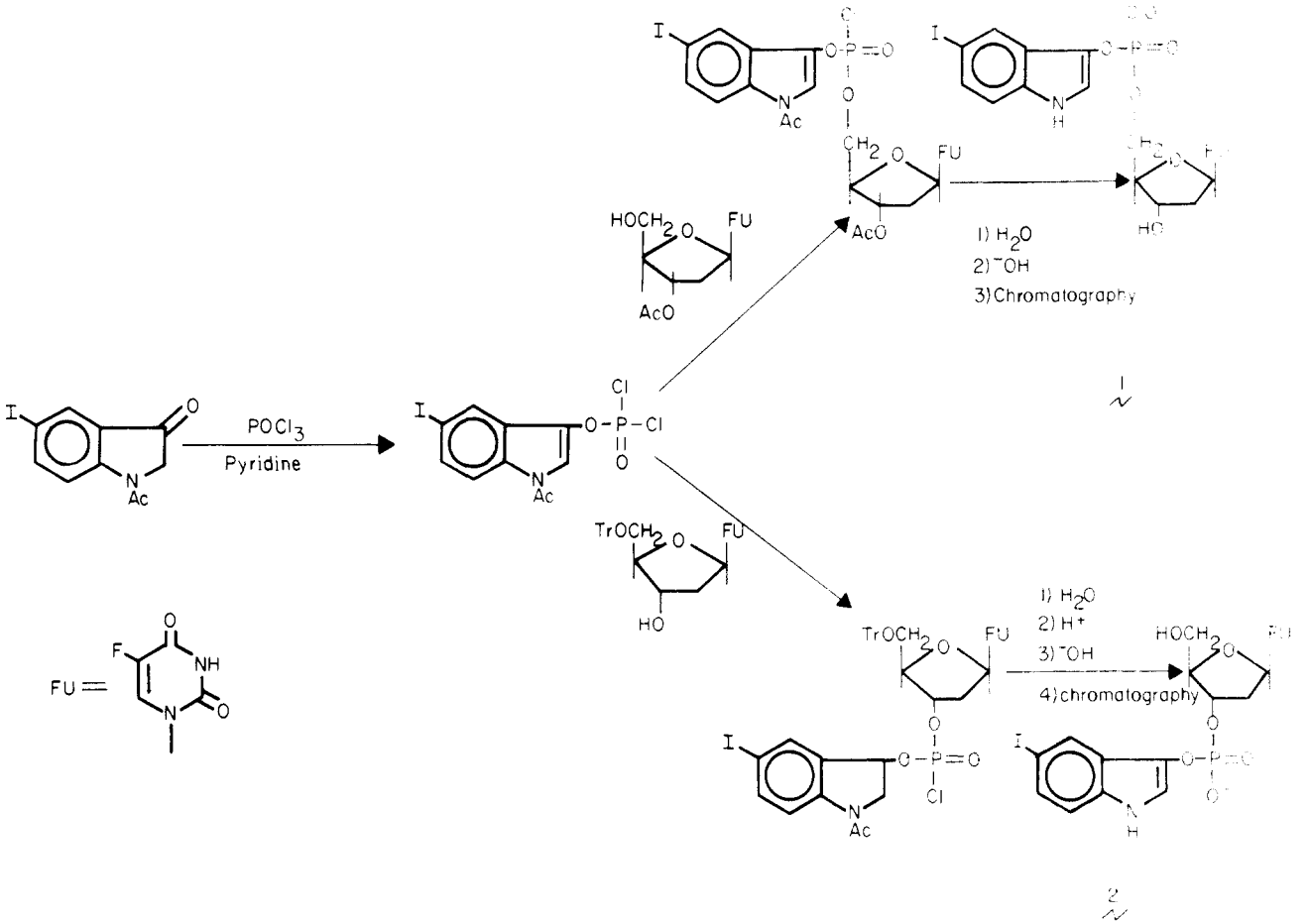
SCHEME II



1010 cm^{-1} which shifts to a weaker band at 1000 cm^{-1}.

Histochemical comparison of these compounds with the deoxythymidine derivatives was made at pH 8.5 and 7.2 with tissues of normal mice and rats. The following tissues were compared: liver, kidney, pancreas, small intestine, and large intestine. Their relative rates are indicated qualitatively in Table I.

TABLE I

RELATIVE RATES OF HYDROLYSIS OF PHOSPHODIESTERS OF FUDR

Tissue	1	2
Liver	++++	0
Kidney	+++	0
Pancreas	++	0
Small intestine	+	0
Rat sarcoma	++	0

Compound **2** was not hydrolyzed by any of the tissues, including spleen. However, purified spleen phosphodiesterase (Worthington) did hydrolyze this compound slowly. Venom phosphodiesterase hydrolyzed **1** very quickly, and formation of indigo dye could be seen in a few minutes.

Several tumors were found to react very readily with **1** to give the indigo dye. They include methylcholanthrene-induced fibrosarcoma, sweat gland tumor, rhabdomyosarcoma of mice, sarcoma of rat, and mouse Glioma-26. Hydrolysis of **1** by Hela cell and mouse Glioma-26 tissue cultures could be observed only after long incubation.

Both **1** and **2** were compared with FUDR in Hela cell tissue culture. Compound **1** was found to be more effective than **2** against this cell line, equal to FUDR at 10^{-5} *M* concentration. Interestingly, only in Hela cells treated with the 5′ derivative could the liberated 5,5′-diiodoindigo be seen. The 3′ derivative was not hydrolyzed. It is thus our opinion that this new chromogenic derivative of FUDR would provide a more direct means of studying the mechanism of FUDR in cancer research. Such an objective has been a long-term goal of developmental enzyme histochemistry in our laboratory and elsewhere.

Experimental Section

5-Fluorodeoxyuridine 5′-(5-Iodo-3-indolyl) Phosphate (dFU-5′-IIP).—3′-O-Ac-dFU[7] (1.06 g, 3.7 mmol) was treated in dry pyridine with 5-iodo-*N*-Ac-indoxyl phosphodichloridate obtained from 606 mg (2 mmol) of 5-iodo-*N*-Ac-indoxyl for 24 hr at room temperature. The reaction mixture was evaporated *in vacuo*, treated with 20 ml of concentrated NH_4OH, left for 24 hr at room temperature and evaporated below 30°. The resultant oil was diluted with H_2O, treated with activated charcoal, and filtered. The filtrate was diluted to 1.6 l. and absorbed on a DEAE-Sephadex column (2.2 × 38 cm). The column was washed with 0.02 *M* NH_4HCO_3 (*ca.* 1.7) and connected to a linear gradient produced from 0.02 *M* (4 l.) and 0.3 *M* NH_4HCO_3 (4 l.). After the developer in the gradient apparatus was used up, additional solution (0.4 *M*, 5 l.) was led to the column. The peak fractions were freeze-dried to yield the almost colorless product, *ca.* 710 mg (61%). After rechromatography, the analytically pure sample was obtained *ca.* 300 mg. *Anal.* ($C_{17}H_{19}FIN_4O_8P$): C, 34.94; H, 3.27; N, 9.59; I, 21.72; P, 5.30. Found: C, 34.87; H, 3.47; N, 9.63; I, 21.45; P, 5.20.

TABLE II
PHYSICAL PROPERTIES OF PHOSPHODIESTERS OF FUDR

Compounds	R_f values[a] Solv 1	Solv 2	Solv 3	Uv spectra[b] λ_{min} (mμ) (ε)	λ_{max} (mμ) (ε)
dT-3′-IIP	0.64	0.68	0.63	250(9200)	268(12600)
dT-5′-IIP	0.59	0.65	0.61	250(9100)	268(12200)
dFU-3′-IIP	0.57	0.71	0.49	251(8300)	271(11800)
dFU-5′-IIP	0.60	0.69	0.45	251(7800)	271(10900)

[a] IIP in solvent 1, 0.65; 2, 0.31; 3, 0.31. [b] All spectra were recorded in distilled H_2O (neutral).

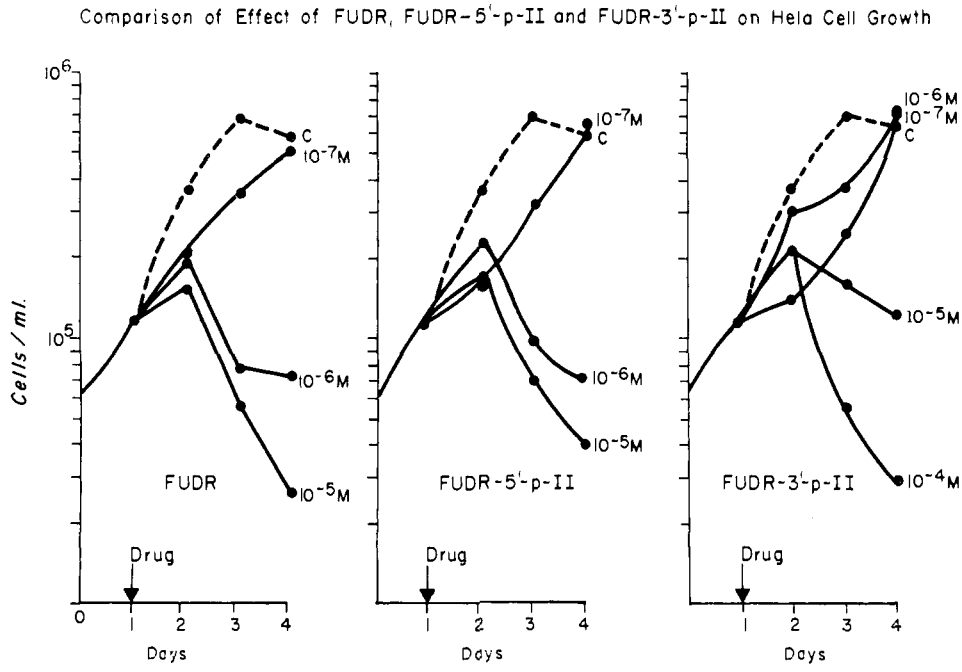


Figure 1.

5-Fluorodeoxyuridine 3′-(5-Iodo-3-indolyl) Phosphate (dFU-3′-IIP).—5′-*O*-Trityl-dFU[8] (2.445 g, 5 mmol) was added to a pyridine solution of the phosphodichloridate (derived from 5 mmol of *N*-Ac-5-iodoindoxyl) and kept at room temperature for 40 hr. The dark reddish reaction mixture was evaporated *in vacuo*, dissolved in $CHCl_3$, treated with an aq solution of $(NH_4)_2CO_3$ (480 mg = 5 mmol), stirred for 0.5 hr, and separated. The $CHCl_3$ phase was washed with 1 *N* NH_4OH, and evaporated to give a dark brown residue. This was dissolved in 35 ml of 80% AcOH and heated in a boiling water bath for 17 min with stirring. After removal of solvent, the residue was treated with 1 *N* NH_4OH, filtered to remove TrOH, and evaporated. The residue was then dissolved in 25 ml of concentrated NH_4OH and left at room temperature for 24 hr. The reaction mixture was evaporated *in vacuo*, dissolved in H_2O, treated with activated charcoal, and filtered. Since the filtrate was still very dark, it was washed again with $CHCl_3$, treated with charcoal, and filtered. This time, the filtrate was much lighter and was then diluted to 3 l. (A_{270} = 10.44, OD_{270} = 31300) and charged on a column (3.2 × 34 cm). After washing with 0.02 *M* NH_4HCO_3, the column was connected to a linear gradient of 0.02–0.3 *M* NH_4HCO_3 (5 l. + 5 l.). Lyophilization of the peak fractions yielded *ca.* 950 mg (32.5%) of slightly brown residue. From rechromatography, an almost colorless fluffy solid (355 gm) was obtained. *Anal.* ($C_{17}H_{19}FIN_4O_8P$): C, 34.94; H, 3.27; N, 9.59; I, 21.72; P, 5.30. Found: C, 35.20; H, 3.40; N, 9.48; I, 21.76; P, 5.29.

Paper Chromatography of 1 and 2.—The R_f values of **1** and **2** in solvent 1 (acidic), BuOH–AcOH–H_2O (5:2:3), solvent 2 (neutral), EtOH–1 *M* NH_4Ac (3:7), and solvent 3 (alkaline), *i*-PrOH–concd NH_4OH–H_2O (7:1:2) are summarized and compared with the deoxythymidine derivative, and also 5-iodoindolyl phosphate (IIP) itself (Table II).

Uv spectra data were obtained with a Beckman DB-G spectrophotometer, and the comparison between the two series is given (Table II).

Histochemical Study.—Fresh-frozen tissue of animals were cut on a cryostat (Pearse). Sections 6 μ in thickness were then incubated in the following media: compounds **1** and **2** (2 mg), and 10 ml of barbital buffer, pH 8.5 and pH 7.0. The sections were then postfixed in 10% formalin for 10 min and examined with a light microscope after mounting in Histoclad. The dye deposits were taken as evidence of enzymatic reaction site.

In a subsequent study, the tissue could be fixed with 10% neutral formalin for 8 hr and kept in gum acacia–sucrose (0.25 *M*) without losing enzyme activity.

All animal tumor tissues were fresh-frozen. Several tumors tested were prefixed. Prostate, stomach, and colon carcinoma all showed positive reactions, but prostate and stomach carcinoma were weakly colored; liver, kidney, and spleen were positive; intestine, relatively weak; and the large intestine was weaker then the small intestine. Specimens were obtained through the Surgical Pathology Laboratory here, and details will be described elsewhere.[11]

Hela Cell Culture Study.—Relative activities of the two compounds, dFU-3′-IIP and dFU-5′-IIP were determined by comparing their effect with that of FUDR on a tissue culture cell line, Hela S-3. Hela cells for assay were grown in Leighton tubes with Eagle's medium (MEM),[12] according to the procedure described by Umeda and Heidelberger.[5] Compounds **1** and **2** were added to the cells after incubation for 24 hr, and their effect was determined quantitatively by cell counts on the 2nd, 3rd, and 4th days, as shown in Figure 1, and by microscopic examination.[13] For counting, cells were removed from the glass by replacing the medium with 0.025% Pronase solution[14] in MEM without serum.

(11) In collaboration with Drs. B. Czernobilsky, H. T. Enterline, and R. G. Ravdin.

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It may be seen that dFU-5′-IIP has a greater activity on these cells than dFU-3′-IIP. Figure 1 permits a comparison of the effectiveness of the compounds on a molar equivalent instead of milligram basis as used in the calculation of ID_{50} values. Comparative ratios (treated cells: control cells) calculated from data obtained on day 3 at a drug concentration of 10^{-6} *M*, are FUDR, 0.11; dFU-3′-IIP, 0.53; dFU-5′-IIP, 0.14 or 11.0%, 53.0%, and 14% inhibition of control cell growth, respectively. Day 3 was chosen because by the 4th day there may be some loss of cells, especially in the control tubes, due to overcrowding and exhaustion of nutrients from the media. In addition, as anticipated theoretically, dFU-5′-IIP was hydrolyzed during cell growth to the purple-blue diiodoindigo which could be observed microscopically inside the cells.

Structure–Antitumor Activity Correlation of Some Schiff Bases

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Schiff bases, containing the >C=N group, are known to have slight antitumor activities;[1] more of these compounds have been synthesized in order to find compounds with greater antitumor activities. The Cancer Chemotherapy National Service Center has screened these compounds against lymphoid leukemia L1210 in the mouse and intramuscular Walker sarcoma 256 in the rat. The antitumor results are reported here with attempts to correlate these activities with the chemical structures of the compounds.

The biological activities of these compounds are difficult to determine because of their rapid hydrolysis in aqueous solution. In some cases the half-life of the uncatalyzed hydrolysis at 25° at pH 7 is only 12 min.[2] Unless the solutions for injections are prepared shortly before use they contain an equilibrium mixture of the Schiff base and its hydrolysis products.

None of the compounds listed in Table I have activity against mouse leukemia L1210. However several of them slow the growth of the intramuscular Walker sarcoma of the rat, in one case to 58% of the tumor growth of the untreated animals. Effectiveness against intramuscular Walter sarcoma of the rat is measured by weights of tumors of treated rats (T) compared with the tumors of control rats (C); the value of T/C must be 0.53 or less for significant activity.[3]

Structure–Activity Relationships.—In applying the Hansch[4] method of correlation to these data the biological activity was expressed in various ways: (1) T/C, (2) C/T, (3) dose in grams for 20% response, (4) log (C/T at the maximum tolerated dose), (5) log (dose in grams for 20% response), (6) dose in moles for 20% response, and (7) log (dose in moles for 20% response). Correlation by each method separately showed the best fit to the data with 1, 2, and 4; log (C/T at the MTD) was chosen because of the nature of Hansch's equation. If more data on the dose–activity curve were available one of the other values probably would have given better results, especially those involving dose levels for a given response. The values in Table I of σ for the *meta* and *para* positions were those compiled by Jaffe[5] and those for the *ortho* position were taken from the work of Barlin and Perrin[6] on the strengths of substituted benzoic acids. Although other values of σ were used in these correlations, these particular values gave the best results. The values of π were taken from a list published by Fujita, *et al.*,[7] based on a study of the partition between 1-octanol and H_2O of 203 mono- and disubstituted benzenes. The values of π in Table I are those for phenoxyacetic acids since these gave the best correlation with our data.

The results of linear regression analysis of each of these variables, individually and collectively, follow (r is the correlation coefficient and s is the standard deviation from regression):

	r	s
$\log C/T = -0.0068\sigma + 0.042$	0.063	0.105
$\log C/T = 0.062\sigma' + 0.0081$	0.537	0.089
$\log C/T = 0.0048\sigma + 0.063\sigma' + 0.0032$	0.539	0.091
$\log C/T = 0.082\pi + 0.045$	0.313	0.100
$\log C/T = -0.150\pi' + 0.00095$	0.531	0.089
$\log C/T = 0.026\pi - 0.138\pi' + 0.0066$	0.539	0.091
$\log C/T = 0.185\pi^2 + 0.005$	0.237	0.103
$\log C/T = 0.463\pi'^2 - 0.048$	0.576	0.086
$\log C/T = 0.239\pi^2 + 0.491\pi'^2 - 0.092$	0.651	0.082
$\log C/T = 0.0062\sigma + 0.065\sigma' + 0.026\pi + 0.017\pi' + 0.0078$	0.546	0.095
$\log C/T = 0.035\pi - 0.079\pi' + 0.284\pi^2 + 0.276\pi'^2 - 0.075$	0.688	0.082
$\log C/T = 0.033\sigma - 0.168\sigma' + 0.054\pi - 0.420\pi' + 0.353\pi^2 + 0.555\pi'^2 - 0.169$	0.747	0.080

The observed values of log (C/T at maximum tolerated dose) in Table I are compared with those calculated from the last equation given above.

The results of linear regression analysis of the 13 Schiff bases of salicylaldehyde (R′ = 2-OH) are given below for comparison:

	r	s
$\log C/T = 0.016\sigma + 0.079$	0.163	0.097
$\log C/T = 0.025\pi + 0.089$	0.102	0.098
$\log C/T = 0.506\pi^2 + 0.018$	0.757	0.064
$\log C/T = 0.029\sigma + 0.062\pi + 0.067$	0.274	0.099
$\log C/T = -0.044\pi + 0.548\pi^2 + 0.013$	0.775	0.065
$\log C/T = 0.015\sigma - 0.022\pi + 0.533\pi^2 + 0.004$	0.786	0.067

Although the correlation coefficients are not remarkably good (r = 1 is perfect correlation), nevertheless

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