Table I. Fertility of Male Rats Treated for 14 Days with Various Chlorine-Substituted Dioxolanes, Dithianes, and Dithiolanes

Compd	Dose, mg/kg	No. males co-habited	No. mated	No. females pregnant	Total implants	No. implants per pregnancy[a]
Propylene glycol		25[b,c]	23	21	265	12.6 ± 0.6
1	5 po	10[b]	9	1	2	2.0
	5 sc	5[b]	4	0	0	0.0
	15 po	13[b]	10	2	2	1.0 ± 0.0
	15 sc	5[b]	4	0	0	0.0
2	5 po	5[b]	2	1	11	11.0
	15 po	5[b]	4	0	0	0.0
	15 sc	5[b]	4	2	32	16.0 ± 2.0
	50 po	5[b]	4	1	1	1.0
	50 sc	5[b]	4	0	0	0.0
3	80 po	5[c]	5	1	2	2.0
	100 sc	4[b]	3	3	11	3.7 ± 1.5
4	80 po	5[c]	5	5	51	10.2 ± 4.1
5	80 po	5[c]	5	5	60	12.0 ± 4.6

[a] Mean ± standard error. [b] Wistar. [c] Sprague-Dawley.

5-Chloro-2-hexyl-1,3-dithiane (5). The same procedure was followed as with **4** except an excess of heptanal (0.18 mol as compared to 0.12 mol of 2-chloro-1,3-dimercaptopropane[11]) was employed. Purification was achieved by column chromatography on Silic AR, CC-7, using ethyl acetate–hexane (1:99) as eluent affording first compound **5** and then 4.6 g of 2-pentyl-2-nonenal. The chromatographed compound **5** was further purified by distillation affording **5** (2.45 g, 9%): bp 89.5° (0.1 mm). *Anal.* ($C_{10}H_{19}ClS_2$) C, H, Cl, S.

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Triphenylmethane Dyes as Inhibitors of Reverse Transcriptase, Ribonucleic Acid Polymerase, and Protein Synthesis. Structure–Activity Relationships

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The discovery of RNA-directed DNA polymerase (reverse transcriptase) activity in RNA tumor viruses[1,2] stimulated an intensive search for inhibitors of this enzyme. It was hoped that such inhibitors might lead to synthesis of drugs that would be useful in the chemotherapy of viral disease and cancer.[3] The anionic triphenylmethane dyes described in this paper represent a class of compounds whose activity against reverse transcriptase has not previously been reported. The structure-activity relationships presented represent a potential starting point for development of new chemotherapeutic agents.

The prototype inhibitor in the triphenylmethane series is aurintricarboxylic acid (ATA, **1**). This dye blocks initiation of protein synthesis in cell-free extracts prepared from bacteria or animal cells[4-6] and the related compound, gallin (**5**), inhibits activity of *Escherichia coli* RNA polymerase.[7] In this paper, we show that these dyes and their analogs are potent inhibitors of a reverse transcriptase prepared from Rauscher leukemia virus. The same compounds were also tested as inhibitors of RNA polymerase activity and of protein synthesis and for their capacity to prevent formation of a DNA-RNA polymerase complex. The essential structure-activity relationships in each of these experimental systems are similar. We also report that Congo Red, ethidium bromide, and 2,6-dimethyl-4-benzyl-4-demethylrifampicin (AF/ABDP)† inhibit protein synthesis in lysates prepared from rabbit reticulocytes.

Structures of dyes used in these experiments are indicated in Figure 1 and Table I. Compounds **1-9** inhibit activity of both polymerases, block formation of the complex between DNA and RNA polymerase, and prevent synthesis of globin (Table II). The most active inhibitors in the triphenylmethane series (**1-8**) inhibit reverse transcriptase activity by 50% at concentrations of 1-2 μM. This concentration is equal or lower than that previously reported for inhibition of this enzyme by compounds **16-18**.[8-10] ATA (**1**) has also been shown to inhibit a highly purified preparation of avian myeloblastosis reverse transcriptase, primed by partially degraded thymus DNA or avian myeloblastosis RNA.[11]

Among triphenylmethane dyes tested, **9** is generally less inhibitory than **1-8**. Aurin (**12**) retained some activity as an inhibitor of globin synthesis; otherwise, **10-13** were essentially inactive as inhibitors in all four assays. Compounds **14-17**, whose inhibitory activity against reverse

† The nomenclature used is that of Gruppo LePetit.

Table I. Dyes and Other Inhibitors

Triphenylmethane dye	Structure	Config[a]	Substituents				
			X	Y[b]	R_1	R_2	R_3
Aurintricarboxylic acid	1	A	COOH		H	COOH	OH
Pyrocatechol Violet	2	A	OH		SO_3H	H	H
Pyrocatechol Violet tetraacetate	3	D					
Gallein	4	B	OH	H	COOH	H	H
Gallin	5	C	OH		COOH	H	H
Gallin tetraacetate	6	C	O-Acetyl		COOH	H	H
Pyrogallol Red	7	B	OH	H	SO_3H	H	H
Bromopyrogallol Red	8	B	OH	Br	SO_3H	H	H
Phenylfluorone	9	B	OH	H	H	H	H
Cresol Red	10	A	CH		SO_3H	H	H
Fluorescein	11	B	H	H	COOH	H	H
Aurin	12	A	H		H	H	OH
Phenol Red	13	A	H		SO_3H	H	H
Other inhibitors							
Congo Red	14						
1, 10-Phenanthroline	15						
Rifampicin	16						
AF/ABDP	17						
Ethidium bromide	18						

[a] See Figure 1. [b] Configuration B only.

transcriptase and *E. coli* RNA polymerase was previously established, serve to compare our results with those reported from other laboratories.[8,19]

Comparison of highly active (1-8) and inactive (10-13) dyes reveals that minimal structural requirements for inhibitory activity in the triphenylmethane series include free or esterified catechol functions in two of the three aromatic nuclei and a sulfonic or carboxylic acid group in the third aromatic ring. A protected sulfonic acid group (*e.g.*, the sulfone in 3) can, apparently, replace the acidic substituent. Judged by the inhibitory activity of 1 and the relative inactivity of 12, the requirement for catechol functions may be satisfied by salicylic acid configurations.

Inhibitory activity was not affected by (a) presence of halogen groups on the rings containing the catechol (8), (b) an ether linkage between the rings (7 and 8), (c) reduction of the quinone structure (5 and 6), or (d) acetylation of the catechol groups (3 and 6). All active dyes except 3 (which may hydrolyze to form a sulfonic acid) are anionic at pH 7.4. The hydroxyl function of 2 cannot be replaced by a methyl group (compound 10). Inactive triphenylmethane compounds (10-13) lack the catechol function; the partially active phenylfluorone (9) lacks the acidic substituent in the third aromatic nucleus.

Of the several chemicals and drugs that inhibit reverse transcriptase,[8,16] many bind to template and are not likely to be selective inhibitors of this enzyme. The rifampicin derivatives may be selective since they do not bind to polynucleotides and the parent compound combines directly with RNA polymerase.[20] We are currently investigating the precise mechanism by which triphenylmethane dyes inhibit reverse transcriptase; by analogy to their action on RNA polymerase, we speculate that the primary effect is exerted on formation of the RNA template-enzyme complex.

In the course of our study, Congo Red, ethidium bromide, and AF/ABDP were found to be potent inhibitors of globin synthesis in reticulocyte lysates. AF/ABDP also inhibits protein synthesis by 50% in intact HeLa cells at a concentration of 15 μM. It is possible that reported antitumor properties of the substituted rifampicins[21,22] in experimental animals are a result of this effect.

Structural requirements among the triphenylmethane dyes for inhibition of globin synthesis, polymerase activity, and formation of the DNA-RNA polymerase complex are similar. We have studied the mode of action of several members of this series in detail. Gallin binds to *E. coli* RNA polymerase, thereby preventing subsequent attachment of DNA and initiation of new chains of RNA.[7] ATA binds to reticulocytes and *E. coli* ribosomes, preventing subsequent binding of messenger RNA.[4-6] This dye also prevents binding of polyuridylic acid to isolated ribosomal proteins[23] and complex formation between bacteriophage $Q\beta$ replicase, *lac* repressor, and their respective templates.[24] We suggest that polynucleotide-protein interactions are selectively blocked by anionic triphenylmethane dyes of the appropriate configuration and that the site(s) on the ribosome that participates in binding mRNA bears similarities to the site on polymerases that binds DNA or other templates.

Experimental Section

Materials. Gallin, gallin tetraacetate, and Pyrocatechol Violet tetraacetate were synthesized by established procedures; the remaining dyes used in these experiments were purchased from commercial sources. Purified ATA, aurin, rifampicin, and AF/ABDP were supplied by Dow Chemical Co. Gallein and phenylfluorone were purchased from K & K Laboratories; Pyrogallol Red, Cresol Red, and Phenol Red from Aldrich Chemical Co.; fluorescein from Mann Research Lab.; Congo Red from Nutritional Biochemicals Corp.; Pyrocatechol Violet from Eastman Organic Chemical Co.; 1,10-phenanthroline from Baker Chemical Co.; and ethidium bromide from Calbiochem.

Enzyme Assays. General Procedures. *E. coli* RNA polymerase was purified by the method of Burgess[25] and assayed with calf thymus DNA template as previously described.[7] Detergent-treated virions of Rauscher leukemia virus (purchased from Electro Nucleonics Laboratories) were used as a source of RNA-directed DNA polymerase; Poly A:oligo dT served as template for this reaction.[26] The assay used was similar to that described by Gurgo, *et al.*,[12] and *Yang, et al.*,[13] in testing effects of various rifamycin derivatives on polymerase activity in Rauscher and murine sarcoma viruses. Crude rabbit reticulocyte lysates were used to measure globin synthesis as previously described.[6] Formation of the complex between RNA polymerase and DNA was determined by the method of Jones and Berg.[27] *E. coli* and ^{14}C-labeled adenovirus DNA were incubated and filtered through nitrocellulose membranes as previously described.[7]

Table II. Effect of Triphenylmethane Dyes and Other Compounds on RNA and Protein Synthesis[a]

Structure	Concentration (μM) of compound required for 50% inhibition: Reverse transcriptase (Rauscher)	*E. coli* RNA polymerase	Globin synthesis	DNA–RNA polymerase complex formation
1	1	2.6	57	12.0
2	1	14.0	25	3.0
3	1	8.0	20	2.5
4	2	10.0	45	8.0
5	2	8.0	96	3.0
6	1	15.0	50	8.0
7	2	2.8	15	0.7
8	2	2.5	7	1.5
9	12	120	150	11
10	>336	>1000	900	>40
11	>336	>1000	>1000	>40
12	>336	1000	130	>40
13	>336	>1000	300	>40
14	4	3.0	3.0	0.2
15	17	>1000	300	>40
16	320	0.1	300	>40
17	50	0.05	13	>40
18	20	14.0	5	>40

[a] Each value represents the average of three separate experiments.

A. Globin Biosynthesis. Lysates were prepared by adding an equal volume of 1 mM $MgCl_2$ to a packed suspension of rabbit reticulocytes and shaking gently for 2 min; cell membranes and debris were removed by low-speed centrifugation.[6] The reaction mixture contained 10 mM Tris-HCl (pH 7.4), 75 mM KCl, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 2 mM magnesium chloride, 2 μM [^{14}C]leucine (315 Ci/mol), 6 mM 2-mercaptoethanol, 0.1 mM mixture of 19 amino acids, 0.9 mg/ml of creatine phosphokinase, 60 μM hemin, 0.01–0.02 ml of lysate, and the specified inhibitor in a final volume of 0.1 ml. Following incubation for 8 min at 33°, 5% TCA was added; the solution was heated for 15 min at 95° and then chilled in an ice bath. Precipitates were collected on Millipore membrane filters and washed three times with cold 5% trichloroacetic acid and the radioactivity was determined on a low-background gas flow counter as described elsewhere.[6] In the control reaction, 3590 cpm were incorporated.

B. RNA Polymerase Activity. The reaction mixture contained 0.04 M Tris buffer (pH 7.9), 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.15 M KCl, 0.5 mg/ml of bovine serum albumin, 46 μg/ml of calf thymus DNA, 0.15 mM concentrations of ATP, GTP, CTP, and [^{3}H]UTP (specific activity, 13.3 mCi/mmol), 20 μg/ml of purified *E. coli* polymerase, and the specified inhibitor in a final volume of 0.25 ml. Reactions were initiated by addition of enzyme; after incubation for 10 min at 37°, the solutions were chilled in an ice bath and 3 ml of 5% trichloroacetic acid solution, containing 0.01 M sodium pyrophosphate, was added. After standing for 15 min at 4°, precipitates were collected on Whatman 2.5-cm GF/C glass filters and washed with a 2% solution of trichloroacetic acid containing 0.01 M sodium pyrophosphate. Radioactivity was determined by liquid scintillation counting in a solution composed of 0.5 ml of Triton and 57 mg of 2,5-diphenyloxazole dissolved in 10 ml of toluene. In the control reaction, approximately 650 pmol of [^{3}H]UMP was incorporated into acid-insoluble material.

C. Reverse Transcriptase Activity. Rauscher leukemia virus was purified by two cycles of banding in sucrose gradients and suspended in 0.01 M Tris-HCl (pH 7.0) to give a final protein concentration of 700 μg/ml. Duplicate enzyme reactions contained 40 mM Tris-HCl (pH 8.3), 60 mM KCl, 5 mM dithiothreitol, 0.025% Triton X-100, 1.5 mM manganese acetate, 0.2 mM [^{3}H]TTP (500 cpm/pmol), 0.35 μg of viral protein, and 1500 pmol of oligothymidylic acid and 7500 pmol of polyadenylic acid as template in a final volume of 0.075 ml. Reactions were incubated for 5 min at 37° before template was added; incubation continued for 1 hr at 37° and reactions were terminated by adding 0.1 ml of 0.02 M sodium pyrophosphate and 0.1 ml of 25% trichloroacetic acid. Precipitates were collected on 0.45-μ Millipore membrane filters, washed with 5% trichloroacetic acid followed by 95% ethanol, and dried, and the radioactivity was determined. In the control reaction, approximately 200 pmol of [^{3}H]TMP was incorporated into acid-insoluble material.

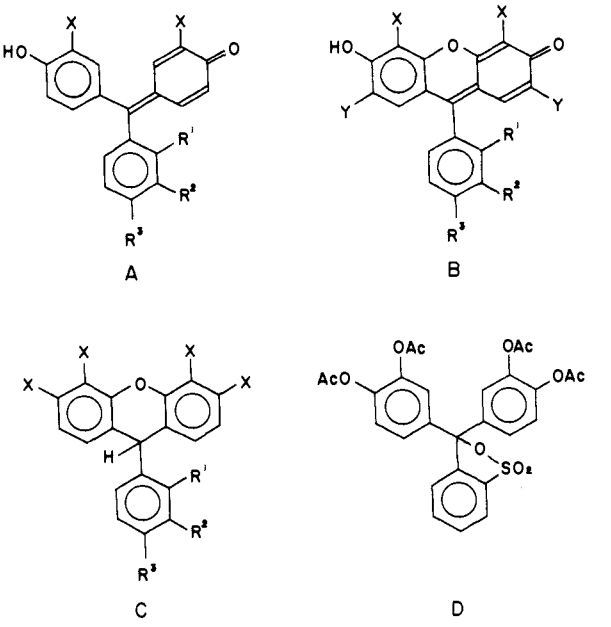

Figure 1. Configurations of triphenylmethane dyes.

D. Formation of RNA Polymerase–DNA Complex. Incubation mixtures contained, in a final volume of 0.25 ml, 0.04 M Tris-HCl (pH 7.9), 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.15 M KCl, 5 μg of RNA polymerase and the specified inhiitor, and 0.25 μg of [^{14}C]adenovirus DNA (3300 cpm). Following incubation for 5 min at 37°, reaction mixtures were diluted with 2 ml of ice-cold 0.01 M Tris (pH 7.9), containing 0.05 M NaCl, and filtered under gentle suction. Filters were washed with 40 ml of Tris-NaCl buffer and radioactivity was determined by liquid scintillation counting as described above. In the control reaction, 2750 cpm remained on the Millipore filter.

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Catechol *O*-Methyltransferase. 5. Structure–Activity Relationships for Inhibition by Flavonoids

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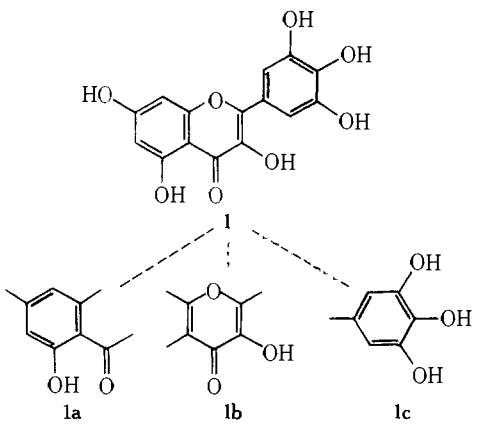
Flavonoids have been long recognized as inhibitors of the enzyme catechol *O*-methyltransferase (COMT,‡ E.C. 2.1.1.6).[1-4] These compounds represent an interesting class of COMT inhibitors because of their multifunctional features which individually would be expected to have the potential of binding to the active site of this enzyme. As an example, myricetin (1) possesses structural features (1a-c) which are present in the COMT inhibitors salicylaldehyde,[5] 3-hydroxy-4-pyrone,[6] and pyrogallol.[7] In an attempt to show the potential involvement of the structural components 1a-c of myricetin (1) in its binding to COMT, we have evaluated as inhibitors of COMT various model compounds in which these three important structural features have been isolated. In this way we have been able to show that there exist three sites on myricetin, which would have the potential to bind to the active site of COMT. The present paper reports the results of this study.



1

1a 1b 1c

Results and Discussion

Table I shows the degree of COMT inhibition produced by the parent compound myricetin (1) and the various model compounds prepared in this study. These model compounds, in which we were able to isolate the various important structural features of myricetin (1), can be divided into three general types: (a) β-hydroxycarbonyl compounds [salicylaldehyde (7), 3,5-dihydroxyflavone (4), and 5-hydroxychromone (5)]; (b) α-hydroxycarbonyl compounds [3-hydroxy-4-pyrone (8), 3-hydroxyflavone (2), and 3-hydroxychromone (3)]; and (c) polyphenolic compounds (pyrogallol). As would be expected chromone (6), which has the basic skeletal structure of myricetin (1), is completely inactive as an inhibitor of COMT. The data in Table I show that the α-hydroxycarbonyl compounds, both 3-hydroxyflavone (2) and 3-hydroxychromone (3), are potent inhibitors of COMT and in fact appear to be more active than 3-hydroxy-4-pyrone (8). This difference in activity probably results from the fact that 3-hydroxy-4-pyrone (8) would show a greater tendency than compounds 2 and 3 to exist in a diketo tautomeric structure.[8] This diketo tautomeric structure would be expected to be inactive as a COMT inhibitor. For the β-hydroxycarbonyl compounds [salicylaldehyde (7), 3,5-dihydroxyflavone (4), and 5-hydroxychromone (5)] relatively weak inhibitory activity toward COMT was observed. Therefore, it would appear that replacement of one of the hydroxyl groups of catechol by a carbonyl function [*e.g.*, salicylaldehyde (7) and 5-hydroxychromone (5)] results in β-hydroxycarbonyl compounds which still have a potential to bind to COMT but provide less than an optimal fit to the enzyme site. In contrast, systems such as tropolone,[9] 3-hydroxy-4-pyridone,[6] and 3-hydroxy-4-pyrone,[6] which are α-hydroxycarbonyl compounds and biochemically isosteric to catechol, appear to bind more tightly to the enzyme. Similarly, systems in which one of the hydroxyl groups of catechol has been replaced by another heteroatom (*e.g.*, 8-hydroxyquinoline[10]) also show a greater affinity for the active site of this enzyme.

Using reciprocal velocity *vs.* reciprocal substrate plots, the kinetic patterns for COMT inhibition by myricetin (1), 3-hydroxychromone (3), 5-hydroxychromone (5), salicylaldehyde (7), and 3-hydroxy-4-pyrone (8) were determined. In order to more accurately compare the inhibitory properties of these compounds with previously reported COMT inhibitors,[5,6,9,10] DHB was used as the catechol substrate rather than *l*-NE. Use of DHB rather than *l*-NE in these kinetic studies also eliminated any possible formation of tetrahydroisoquinolines by reaction of *l*-NE and

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‡Abbreviations used are COMT, catechol *O*-methyltransferase; SAM, *S*-adenosyl-L-methionine; DHB, 3,4-dihydroxybenzoate; *l*-NE, *l*-norepinephrine; K_{is}, inhibition constant for the slope; K_{ii}, inhibition constant for the intercept.